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56. THE NUTRITIVE VALUE OF PROTEINS FOR MILK PRODUCTION

I. A COMPARISON OF THE PROTEINS OF BEANS, LINSEED, AND MEAT MEAL

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(With 5 Figures.)

CONTENTS.

	PAGE
Introduction	177
Experimental methods	179
(*a*) Animals	179
(*b*) Rations	179
(*c*) Technique of collection and separation of excreta	181
(*d*) Sampling and analysis	181
Discussion of results	181
(*a*) Milk yield	181
(*b*) Total nitrogen of milk and urine	183
(*c*) Nitrogen partition in the urine	187
(*d*) Total output of milk, urine and faeces	189
(*e*) Faecal nitrogen	189
(*f*) Nitrogen balances	190
(*g*) Calculation of biological values	191

INTRODUCTION.

THE protein requirements for maintenance and milk production are based on the well-known investigations of Kellner, Armsby, Haecker, Savage and others, who recommend feeding standards varying from 0·5 to 0·8 lb. digestible crude protein per 1000 lb. live weight for maintenance, and from 0·52 to 0·63 lb. digestible crude protein per 10 lb. milk for milk production. A detailed study of the work of these investigations shows that in many instances nitrogen equilibrium has been obtained on considerably lower planes of protein intake: but the figures have been arbitrarily raised, partly to provide a sufficient safeguard against underfeeding, and partly on the assumption that the excess food protein could exert a stimulating effect on mammary secretion. Recent investigations, particularly those of Hills and his associates (1), Perkins (2), Buschmann (3) and Savage and Harrison (4) indicate that the existing protein standards could, in fact, be considerably reduced. The above investigations have, however, been limited to a study of the *quantity* of protein required for maintenance and milk production, and no account has been taken of the possible effect of variations in the *quality* of proteins on their utilisation.

The specific function of protein in animal nutrition is to provide the body with adequate quantities of available nitrogen, but it is essential that part of this nitrogen should be supplied in the form of certain amino acids which are indispensable constituents of the tissues and secretions, but which the body itself is unable to synthesise. Chief among these amino acids are lysine (5), tryptophane (6), cystine (7), and arginine and histidine (8). It is obvious therefore that the efficiency with which a given food protein will meet the requirements of any specific function, such as growth or milk secretion, will depend on the extent to which it is able to supply these essential amino acids in adequate quantities and in the correct proportions.

In the present investigation an attempt has been made to study this question by determining the comparative values of the proteins of beans, linseed and meat meal for milk production. It will be seen from Table I[1] that, when compared with the protein of bean meal, the proteins of linseed contain a relatively small quantity of lysine, while those of meat meal are seriously deficient in tryptophane.

Table I. *Amino-acid content of foods (as percentage of total N).*

	Lysine	*Tryptophane	Cystine	Histidine *plus* arginine
Separated milk	8·34	4·54	2·04	15·32
Bean meal	7·45	2·29	1·00	13·20
Linseed-oil meal	*3·34*	2·80	1·00	21·51
Linseed cake	*4·21*	2·87	1·14	20·87
Meat meal	8·80	*0·63*	1·40	20·94

* This figure represents insoluble humin, and can only be taken as a very rough estimate of the tryptophane content.

Since the proportion of these two amino acids in milk is high, it would be expected that with a ration containing bean meal there would be a more efficient utilisation of protein than with a similar ration containing either linseed or meat meal.

In carrying out an experiment of this nature it is, however, essential that the food intake should contain minimal quantities of protein: otherwise the amino-acid deficiency on the rations containing poor quality protein will not become apparent. Thus, if the normal protein standard for milk production (0·6 lb. dig. protein per 10 lb. milk) were used, there would either be a danger of obtaining a high wastage—which would invalidate calculations of the biological value of the food proteins (9), or the effect of the deficient proteins on milk yield would be masked.

In previous investigations this precaution has, in fact, been overlooked. Thus, while Hart and Humphrey (10), in a series of feeding trials involving a comparison of milk, corn, wheat, and oil meals for milk production, were able to demonstrate differences between these foods, their data cannot be used

[1] The figures in Table I were obtained by a modification of Van Slyke's nitrogen distribution method. A full description of the method and results will be published in a later issue of the *Journal*.

for the calculation of the biological values of the food proteins on account of the high nitrogen intake. Similarly Zorn and Richter (11), in a comparison of the proteins of blood meal and earthnut cake, were unable to demonstrate any adverse effect of the latter on milk yield, although it appears from amino-acid analyses, that the proteins of earthnut cake are seriously deficient in lysine. In the present investigation this error has been largely avoided by feeding a production ration containing only 0·44 lb. protein equivalent[1] per 10 lb. milk.

EXPERIMENTAL METHODS.

(*a*) *Animals.*

Four milking cows were used in the experiment, each animal being at the same stage of lactation, *i.e.* 4–6 weeks from calving. None of the cows was in-calf during the experiment. The following are the details of each animal:

Animal	Breed	Age (years)	Weight (lb.)	No. of lactations	Date of calving	Date of commencement of exp.	Milk yield at commencement of exp. (lb. per day)
Mona	Ayrshire	$11\frac{9}{12}$	1042	8	2. xi. 31	2. xii. 31	$37\frac{1}{2}$
Butterfly	,,	$4\frac{1}{12}$	985	2	28. xi. 31	3. i. 32	$37\frac{1}{2}$
Milkmaid	,,	$4\frac{9}{12}$	912	3	5. ix. 32	14. x. 32	39
Kate	,,	$4\frac{3}{12}$	934	?3	22. viii. 32	14. x. 32	35

(*b*) *Rations.*

Prior to the commencement of the experiment the feed of the four cows consisted of a maintenance ration of oats, beans, linseed and meat meal, in the ratio 2 : 1 : 1 : 0·1.

Each animal was under experiment for four periods of 4 weeks. In the first three periods the three concentrates—beans, linseed, and meat meal—were alternated, the ration for the fourth period always being identical with that for the first period.

Throughout the experiment each cow received a maintenance ration of straw, beet pulp and oats, the combined ration being fed at the rate of 6 lb. starch equivalent and 0·6 lb. protein equivalent per 1000 lb. live weight. As already stated, the production ration was fed at the rate of 0·44 lb. protein equivalent per 10 lb. milk, while the starch equivalent was retained at the usual figure of 2·5 lb. per 10 lb. milk. It was therefore impossible for the production ration to be made up solely of the selected concentrates (beans, linseed, or meat meal), and a basal quantity of oats was added in order to adjust the energy content of the ration. If necessary, starch was also added.

The three concentrates were fed in the production ration in such quantities that each supplied equivalent amounts of nitrogen to the ration. For the feeding of the first two animals (Mona and Butterfly) the linseed was supplied as linseed-oil meal, pure linseed being ground on the farm. As it

[1] Protein equivalent $= \frac{\text{dig. true protein} + \text{dig. crude protein}}{2}$.

appeared that the high content of oil in this product might affect milk yield, linseed cake was used in the rations of the second two animals (Kate and Milkmaid).

The production ration was based on the initial milk yield, and was not reduced as lactation advanced or as milk yield fell. It will be seen, therefore,

Table II. *Details of daily rations.*

Mona

Maintenance
4·550 kg. straw
1·500 kg. beet pulp
1·700 kg. oats

Production
5·100 kg. oats
plus
Period 1: 1·500 kg. bean meal, 1·045 kg. starch
Period 2: 1·617 kg. linseed-oil meal
Period 3: 0·500 kg. meat meal, 1·412 kg. starch
Period 4: same as (1)

Butterfly

Maintenance
As for Mona

Production
5·100 kg. oats
plus
Period 1: 1·617 kg. linseed-oil meal
Period 2: 1·500 kg. bean meal, 1·045 kg. starch
Period 3: 0·500 kg. meat meal, 1·412 kg. starch
Period 4: same as (1)

Kate

Maintenance
3·413 kg. straw
1·500 kg. beet pulp
1·700 kg. oats

Production
5·100 kg. oats
plus
Period 1: 1·500 kg. bean meal
Period 2: 1·300 kg. linseed cake
Period 3: 0·500 kg. meat meal
Period 4: same as (1)

Milkmaid

Maintenance
As for Kate

Production
4·617 kg. oats
plus
Period 1: 1·300 kg. linseed cake
Period 2: 1·500 kg. bean meal
Period 3: 0·500 kg. meat meal
Period 4: same as (1)

Table III. *Lysine and tryptophane content of total rations (as percentage of total N fed per day).*

	Mona		Butterfly		Kate		Milkmaid	
Period	Lysine %	Trypto-phane %	Lysine %	Trypto-phane %	Lysine %	Trypto-phane %	Lysine %	Trypto-phane %
1	3·60	4·55	*2·58*	4·68	3·65	4·30	*2·80*	4·48
2	*2·58*	4·68	3·60	4·55	*2·80*	4·48	3·67	4·31
3	3·80	*4·27*	3·80	*4·27*	3·80	*4·00*	3·87	*4·00*
4	3·60	4·55	*2·58*	4·68	3·65	4·30	*2·80*	4·48

(*Note.* Amino-acid deficiencies are shown in italics.)

that in each experimental period the total nitrogen content of the ration was identical, the only difference between the periods being in the *quality* of the protein fed. The actual quantities of food and order of feeding periods are shown in Table II, while Table III shows the lysine and tryptophane contents of the complete rations. Water was allowed *ad lib.*, but the water-bowls were provided with metres to allow measurement of water consumption.

(*c*) *Technique of collection and separation of excreta.*

The animals were housed in special metabolism stalls which permitted accurate feeding and which were fitted with an apparatus which enabled the urine and faeces to be collected and separated automatically. A full description of this apparatus is given by Ritzman and Benedict (12). Briefly, the excreta falls or is directed through an opening in the floor on to a slowly moving endless belt. This belt is carried on rollers, one of which is set somewhat higher than the other, the direction of movement of the belt being on an incline and uphill. Faeces lodge on the belt and are carried uphill, where they are removed by a scraper and fall into a special container. Urine flows down the belt, or over the sides, and is caught in a large funnel which leads to the collecting bottle. The apparatus, which is, of course, kept running both day and night, has been found most satisfactory, the separation of urine and faeces being practically perfect.

(*d*) *Sampling and analysis.*

The sampling of urine presents no difficulties. On the other hand very considerable errors are possible in sampling from large quantities of faeces unless care is taken to ensure thorough mixing of the whole mass. In the present experiments the collected faeces were thoroughly mixed to a homogeneous mass in a rotary mechanical mixer before sampling. Samples of urine and faeces were taken every second day for the last 14 days of each experimental period. The milk was weighed and sampled after each milking.

Faeces and milk were analysed for total N, and urine for total N and also for partition products. The methods used were: total N (Kjeldahl), urea and ammonia (Folin aeration), creatinine and creatine (colorimetric), amino-acid N (Sörensen's formal titration).

DISCUSSION OF RESULTS.

(*a*) *Milk yield.*

Since the quantities of protein fed in the present experiment were minimal—that is, since even on the good quality protein (bean meal) the amounts were scarcely adequate to supply the requirements for milk production—the feeding of a protein deficient in essential amino acids would be expected *either* to lower milk yield, *or* to cause the animal to draw on its body reserves until these were exhausted, when a fall in milk yield would take place. The results shown in Fig. 1 bear out this expectation.

In this figure the lactation curves for each of the four experimental animals are given, the change from one feeding period to another being marked by a vertical line. The dotted line shows the expected lactation curve for an animal fed throughout the experiment on the adequate protein (bean meal). It will be seen that with the two cows, Mona and Kate, who commenced on bean-

meal rations, a change to linseed caused a distinct fall in milk yield, the fall being more marked with the oil meal than with the cake. In the subsequent change to meat meal, there was at first a tendency towards improved yield (particularly with Mona), but the yield fell away at the end of the period. The final period on bean meal showed, after a short time, a complete return to the normal level. With the two cows, Butterfly and Milkmaid, who commenced on linseed rations, similar results were obtained. During the first linseed period the milk yield was definitely subnormal, the fall in yield being again more marked with the oil meal than with the cake. The change to bean

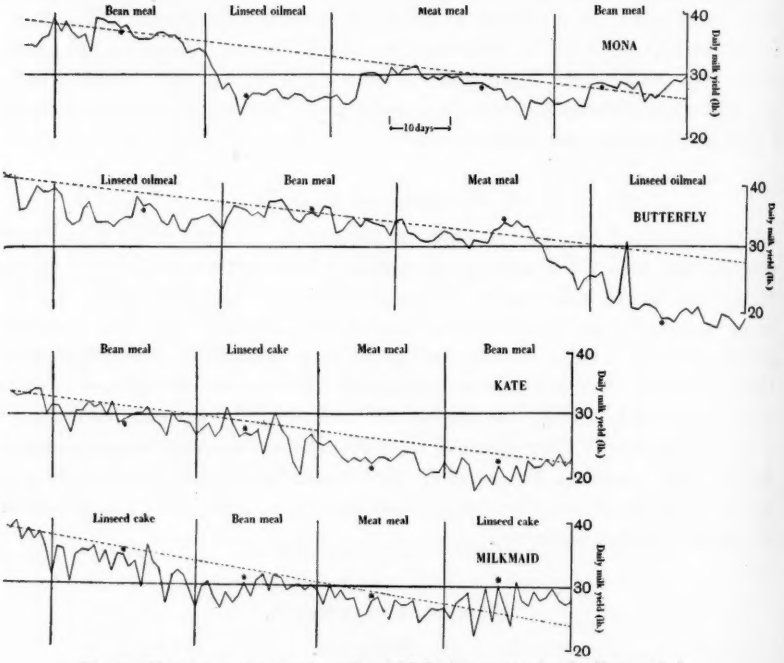

Fig. 1. Showing variations in milk yield during successive feeding periods.

(*Note.* Asterisk indicates commencement of 14-day periods during which complete nitrogen metabolism was studied.)

meal was accompanied by a gradual return to the normal level. The meat meal period showed a slight falling off in yield, which was most marked towards the end of the period. During the final linseed period, Butterfly's yield showed a drastic fall. On the other hand, Milkmaid's yield rose remarkably, an anomalous result which will be referred to in a later section (p. 191).

It will be seen that there is a distinct parallelism in the results of all four cows, with the one exception noted above. It is evident that the fall in milk yield which occurs with linseed oil meal is greater than that which is found

when the cake is fed. This difference is probably due to the high oil content of the meal, since it is well known that excess fat tends to decrease milk yield. Thus Kellner (13) reported a decreased yield with increased fat feeding on a series of experiments involving 196 cows, while Sheehy (14) and Brown and Sutton (15) found the same effect with linseed and menhaden oils. Even when this factor is taken into account, however, it is apparent from the results that the yields on the two deficient rations (linseed and meat meal) are significantly lower than those on the adequate ration (bean meal).

Moreover a careful examination of the curves for each feeding period provides evidence of the storage of reserve N which can be utilised for milk production when the food protein is inadequate. Thus it will be seen that, with both the linseed and meat meal rations, the fall in milk yield does not take place immediately the ration is fed, but there is a preliminary period varying from 4 to 18 days during which the yield is maintained at a normal level. Similarly, when the feed is changed from a deficient ration (linseed or meat meal) to an adequate ration (bean meal), the return to normal yield is not immediate, but may take from 6 to 18 days. The prolonged period between the change in ration and the fall from, or return to, the normal lactation curve cannot be due to the slow removal of the previous feed from the gut, since Ewing and Smith (16) have shown that food will pass through the alimentary tract within from 72 to 84 hours. It appears, therefore, that the delayed reaction of the milk yield to dietary changes must be due in the one case to the utilisation, and in the other to the formation, of reserve N in the body. There is no evidence to show whether the nitrogen storage takes the form of protein (for instance, Rubner's (17) "transitional protein") or whether, as Folin and Denis (18) hold, the reserve material is non-protein in character, though there is definite evidence (p. 188) that tissue metabolism is increased during the feeding of the deficient rations. The demonstration of the utilisation of reserve N is, however, of fundamental importance in any study of the comparative values of food proteins, since it indicates that, with short-period feeding experiments, the conclusions may be entirely erroneous. Unfortunately in the present experiments metabolism studies were not made during the change-over periods, so that additional evidence on this point is not available. A further series of experiments is at present in progress in order to elucidate this question.

(*b*) *Total nitrogen of milk and urine.*

Figs. 2, 3, 4 and 5 show the relation between the total N secreted in the milk during the four experimental periods, and the total urinary N. It should be noted that these and subsequent results refer only to the last 14 days of each feeding period.

In general it may be assumed that total urinary N represents the wastage in nitrogen which occurs either as a result of overfeeding, or as a consequence of feeding a source of nitrogen which cannot be utilised by the body. This

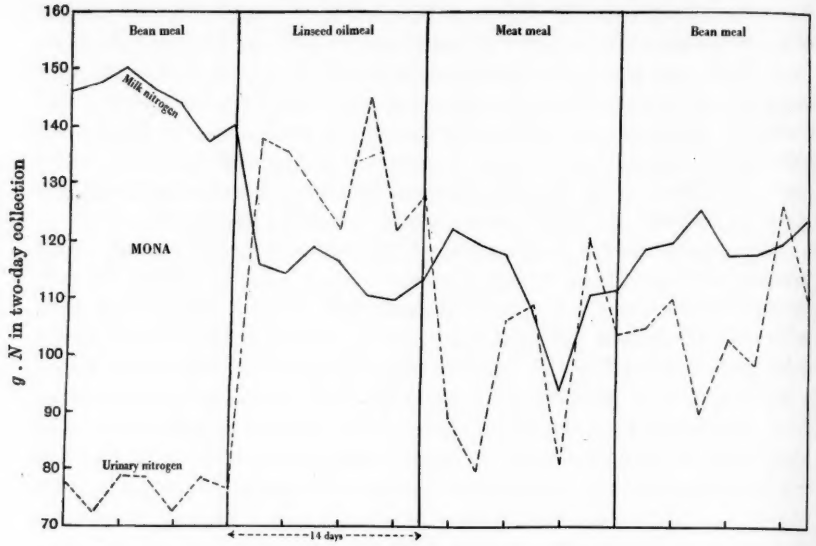
Bean meal
Linseed oilmeal
Meat meal
Bean meal
Milk nitrogen
MONA
Urinary nitrogen
14 days
g . N in two-day collection
160
150
140
130
120
110
100
90
80
70

Fig. 2.

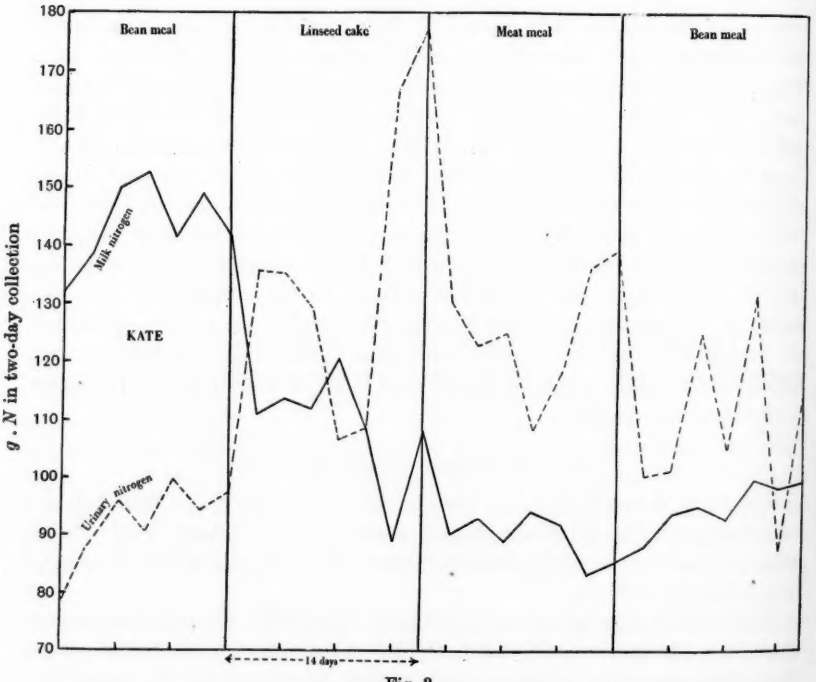
Bean meal
Linseed cake
Meat meal
Bean meal
Milk nitrogen
KATE
Urinary nitrogen
14 days
g . N in two-day collection
180
170
160
150
140
130
120
110
100
90
80
70

Fig. 3.

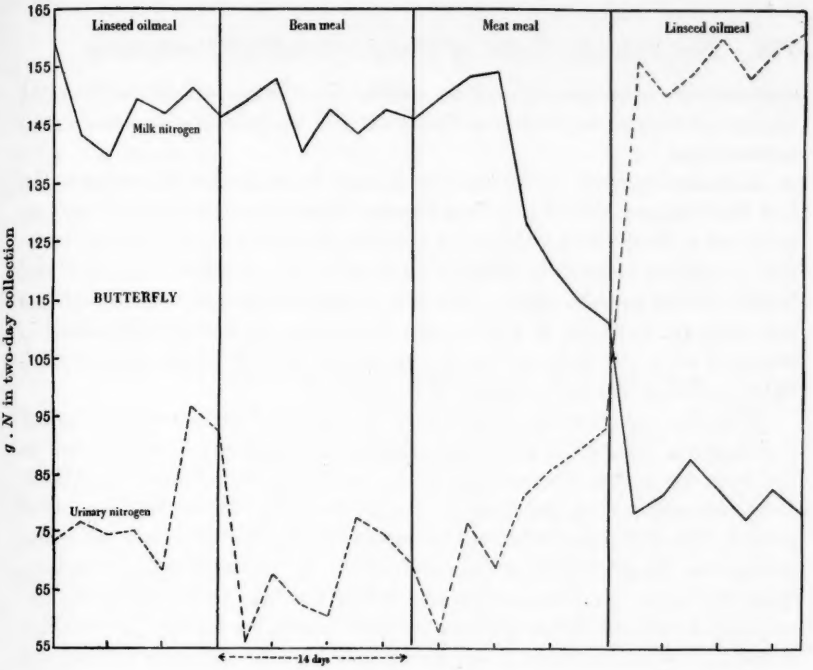
Linseed oilmeal
Bean meal
Meat meal
Linseed oilmeal
Milk nitrogen
BUTTERFLY
Urinary nitrogen
g . N in two-day collection
165
155
145
135
125
115
105
95
85
75
65
55
14 days

Fig. 4.

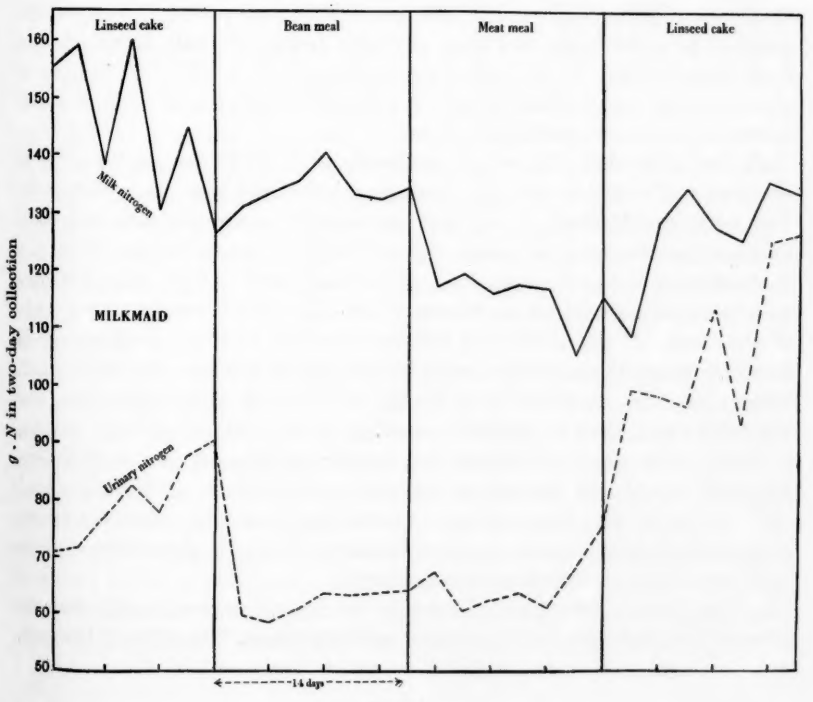
Linseed cake
Bean meal
Meat meal
Linseed cake
Milk nitrogen
MILKMAID
Urinary nitrogen
g . N in two-day collection
160
150
140
130
120
110
100
90
80
70
60
50
14 days

Fig. 5.

statement is, of course, subject to qualification, since part of the excreted nitrogen is endogenous in origin. This point will be dealt with in detail in the next section.

In interpreting the present results it must be realised that, owing to the fact that the quantity of protein fed remained constant throughout each experiment while the milk yield would normally fall off slowly throughout lactation, a gradual increase in wastage would be expected between the first and fourth feeding periods. Even when this normal increase in wastage is taken into account, however, it will be seen that there are marked alterations in the total urinary N between the various feeding periods which must be attributed to differences in the nature of the rations.

With the two cows (Mona and Kate) who received bean meal for the first period (Figs. 2 and 3), it will be seen that the total urinary N remains low on the bean meal, rises dramatically in the linseed period, falls off slightly in the meat-meal period, and finishes at a medium level in the final bean-meal period. The difference between the values in the first and last bean-meal periods can be attributed to the overfeeding, and consequent high wastage, which will have been attained by the end of the final period. With the two cows (Butterfly and Milkmaid) who received linseed for the first period (Figs. 4 and 5), the total urinary N was moderately high for the linseed period, fell in the bean period, rose gradually in the meat-meal period, and ended at a very high level in the final linseed period. Allowance should again be made for the overfeeding which takes place towards the final period. An important point to be noted is the fact that, although Milkmaid's milk N rises in the final linseed period (an anomalous result already referred to), the urinary N also rises markedly, indicating that, in spite of the higher milk yield, wastage is taking place on a considerable scale.

Taken as a whole, the above results show (*a*) that bean-meal feeding is accompanied by a low nitrogen wastage, (*b*) that the wastage is extremely high on linseed feeding, and (*c*) that the wastage on meat-meal feeding falls between the two extreme values. These differences can be explained on the basis of the amino-acid composition of the food protein. Cary (19) and Blackwood (20) have shown that the precursors of milk protein are the amino acids of the blood. If the amino-acid mixture resulting from the food protein is correctly proportioned for the synthesis of milk protein (as with bean meal) utilisation of the assimilated amino acids will be high, and wastage low. On the other hand, if an amino acid, essential for the synthesis of milk protein, is lacking from the food protein (*e.g.* lysine from linseed, and tryptophane from meat meal) the amount of this amino acid assimilated from the food will be the limiting factor in milk protein synthesis, the remaining amino acids will be assimilated in excess of requirements, and a proportion of these will therefore be excreted as waste products.

It has already been pointed out that the body may temporarily draw on reserve N to make good such an amino-acid deficiency. This will not, however,

affect the total urinary N output, since, from whatever source the missing amino acid is supplied, there will be a concurrent liberation of other amino acids in excess of requirement. This point is well illustrated in the high wastage occurring in Milkmaid's fourth feeding period, where, although reserve N is used for milk production (p. 188), a high total urinary N is found.

(c) *Nitrogen partition in the urine.*

Figures for the total urinary N and the N partition products averaged for each feeding period of 14 days, are shown in Table IV.

Table IV. *Urinary excretion of nitrogen (average 2-day output for each feeding period).*

Cow	Period	Urea N g.	Ammonia N g.	Amino-acid N g.	Creatinine N g.	Creatine N g.	Total N g.
Mona	Beans	47·04	9·39	7·30	8·18	4·33	76·31
	Linseed	67·80	39·89	6·02	9·06	6·98	135·12
	Meat meal	60·49	21·89	1·54	13·96	8·61	98·18
	Beans	60·74	20·50	1·83	10·20	5·51	105·92
Butterfly	Linseed	47·44	19·10	2·02	6·58	6·53	79·44
	Beans	*32·83	*17·36	2·51	7·68	4·94	67·02
	Meat meal	42·44	20·66	1·63	9·73	6·67	79·08
	Linseed	83·19	47·19	1·78	8·29	6·20	156·22
Kate	Beans	60·06	8·50	9·54	12·38	4·05	92·42
	Linseed cake	75·14	32·52	11·26	10·37	6·46	137·21
	Meat meal	78·04	17·62	8·82	13·72	8·81	125·82
	Beans	72·87	12·07	9·58	12·58	4·80	109·60
Milkmaid	Linseed cake	41·87	21·65	9·00	8·53	5·64	79·52
	Beans	37·18	9·30	5·18	7·42	4·28	61·92
	Meat meal	36·05	8·42	4·32	10·27	5·67	65·43
	Linseed cake	56·32	24·10	10·83	8·98	9·30	107·49

* Urine contaminated due to fault in sieve.

The chief products of exogenous N metabolism are urea and ammonia. It will be seen from Table IV that these two products account for the larger part of the very considerable differences in total urinary N which occur in the various feeding periods. The results therefore confirm the suggestion put forward in the previous section that the feeding of a protein deficient in essential amino acids will lead to a marked increase in the excretion of exogenous metabolites through the deamination of the excess circulating amino acids.

Table V shows the partition products expressed as percentages of the total urinary N. With one exception (Butterfly), the results demonstrate that when linseed is fed the percentage of urea N shows a slight fall, although the total output is, of course, increased. Meat meal also shows a decreased percentage as compared with beans, but an increase over linseed. The ammonia N, on the other hand, shows a remarkably increased output, both total and percentage, when linseed is fed, the percentage output being more than doubled in certain instances. The results for meat-meal feeding are variable, but, except in one instance, are not significantly higher than for beans. Little significant change is found in the excretion of amino-acid N.

The high output of ammonia N on the linseed would normally be taken as a symptom of marked acidosis. Such an acidosis might possibly arise from the excessive deamination of amino acids, leading to the formation of large quantities of organic acids according to the reaction:

$$R.CH.NH_2COOH + H_2 \rightarrow R.CH_2COOH + NH_3.$$

Alternatively it might be due to the excessive production of organic acids in the alimentary tract from the carbohydrate moiety of the linseed. If the feeding of linseed does, in fact, cause a marked acidosis, it would appear that the prolonged feeding of rations containing linseed might exert a detrimental effect on the body. The present results must, however, be regarded as tentative, though they are sufficiently suggestive to warrant further investigation.

With regard to endogenous metabolism, this is represented in Table IV by the creatinine and creatine output. The creatinine excretion is fairly con-

Table V. *Urinary partition of nitrogen (expressed as percentage of total urinary N).*

Cow	Period	Urea N	Ammonia N	Amino-acid N
Mona	Beans	61·5	12·3	9·6
	Linseed	50·0	29·6	4·4
	Meat meal	61·5	22·3	1·6
	Beans	57·2	19·3	1·7
Butterfly	Linseed	59·6	24·1	2·5
	Beans	*49·0	*25·8	3·7
	Meat meal	53·6	26·2	2·2
	Linseed	53·1	30·2	1·1
Kate	Beans	65·0	9·2	10·3
	Linseed cake	54·6	23·7	8·2
	Meat meal	62·0	14·0	7·0
	Beans	66·2	11·0	8·7
Milkmaid	Linseed cake	52·6	27·6	11·4
	Beans	60·0	15·0	8·3
	Meat meal	55·4	12·9	6·8
	Linseed cake	52·4	22·4	10·0

* Urine contaminated due to fault in sieve.

stant, but the figures for the meat-meal periods are invariably higher than those for either the bean or linseed periods. This is probably due to the presence of creatinine in the ingested meat meal. On the other hand the creatine figures show well-marked variations according to the ration fed. Low values (*circa* 4·5 g.) are invariably obtained during the bean-meal periods, but a change to either linseed or meat meal causes a marked increase which may vary from about 25 per cent. to over 100 per cent. These results demonstrate clearly that, during the feeding of the deficient proteins, a marked muscular catabolism is present. It has already been noted that during these feeding periods the animal is attempting to maintain the milk yield by the utilisation of her own body tissue, and the creatine results provide a striking confirmation of this fact. The most marked example is that of Milkmaid in her final feeding period. During this period the milk yield rose substantially above the level of the preceding meat-meal period. In the previous section

it has been shown that this increase in milk yield was accompanied by a marked increase in total urinary N. The present results show that an intense tissue catabolism, as shown by an increase in creatine excretion from 4·2 to 9·3 g., was also present.

The creatine figures thus provide a clear demonstration of the utilisation of reserve N by the body in its attempt to maintain milk production on a deficient protein ration.

(d) Total output of milk, urine and faeces.

In Table VI the intake of food and water for each period is compared with the total output of milk, urine and faeces. These figures have little significance as regards the comparative values of the different rations, but they are included in order that complete data on the present experiments may be available.

Table VI. *Comparison of total intake with total output (2-day average for each feeding period).*

		Intake		Output		
Cow	Feed	Quantity g.	Water gal.	Urine c.c.	Faeces g.	Milk lb.
Mona	Beans	15,395	18·3	7,457	57,743	71·4
	Linseed	14,467	18·5	13,257	53,643	53·3
	Meat meal	14,762	18·7	10,136	57,950	53·2
	Beans	15,395	17·2	11,386	53,350	56·1
Butterfly	Linseed	14,467	19·3	5,534	51,380	68·6
	Beans	15,395	19·3	6,843	52,607	67·8
	Meat meal	14,762	15·0	6,571	41,550	58·5
	Linseed	14,467	15·1	6,129	44,380	38·0
Kate	Beans	12,530	16·6	5,079	49,622	58·9
	Linseed cake	12,730	15·9	8,114	51,524	52·8
	Meat meal	11,730	13·0	8,550	60,550	45·3
	Beans	12,530	14·8	7,036	48,757	43·1
Milkmaid	Linseed cake	13,213	16·0	7,864	45,500	63·9
	Beans	13,013	14·4	4,901	52,480	59·5
	Meat meal	12,213	16·0	4,260	61,399	52·8
	Linseed cake	13,213	10·6	6,086	45,000	55·6

(e) Faecal nitrogen.

Figures for the output of nitrogen in the faeces are given in Table VII. The outstanding fact revealed from these figures is the decrease in faecal N

Table VII. *Variation in nitrogen output of faeces (average 2-day output for each feeding period).*

Cow	Feed	Faecal N g.	Cow	Feed	Faecal N g.
Mona	Beans	185·44	Kate	Beans	140·42
	Linseed	149·31		Linseed cake	121·36
	Meat meal	178·76		Meat meal	167·99
	Beans	173·52		Beans	155·65
Butterfly	Linseed	133·33	Milkmaid	Linseed cake	135·79
	Beans	155·97		Beans	162·60
	Meat meal	144·12		Meat meal	*213·87
	Linseed	127·33		Linseed cake	131·20

* Abnormal nitrogen metabolism through injury.

which occurs during the linseed feeding periods. At first sight it seemed possible that this might be due to normal variations in digestibility, but an examination of published digestibility figures showed that this explanation was not tenable.

	Beans	Linseed-oil meal	Linseed cake	Meat meal
% crude protein digestible	80·0	79·2	86·2	93·0

A further explanation might be that the consistency of the rations containing linseed was looser than that of rations containing bean meal or meat meal, and that this would result either in a lowered excretion of metabolic N, or in an increased digestibility of the other constituents of the ration. While, however, the consistency of the faeces was relatively softer on the linseed-oil meal, on linseed cake the faeces retained their normal firmness.

Failing an explanation on these lines, the possibility must be considered that the body itself can to some extent adjust the assimilation of nitrogen from, or re-excretion of nitrogen into the gut according to its needs. In this connection it should be pointed out that the proteins fed in the present experiments were inadequate, on the deficient rations, to supply the requirements for normal milk production, and that the body was already utilising its reserve N for this purpose. It is therefore not unreasonable to assume that absorption from the gut might be stimulated when inadequate rations were fed. The present results suggest that an investigation might usefully be undertaken into the effect of varying levels of feeding on the digestibility of food constituents.

(*f*) *Nitrogen balances.*

It is well known that the resting animal tends to retain N, the period of retention depending on the previous body activity. In other words, the maintenance requirement of the resting animal is a fraction of that of an animal allowed free movement. This is illustrated in the varied figures given by different workers for the maintenance requirements of cows. Halnan (21), for instance, in a review of the subject, quotes figures varying from 0·28 to 0·70 lb. digestible crude protein per 1000 lb. live weight.

In the present experiments the cows were given a maintenance ration containing 0·6 lb. protein equivalent per 1000 lb. live weight—the standard normally allowed for the maintenance of dairy cows. Yet, with two exceptions, positive balances were noted in every feeding period (see Table VIII). Since the production ration only supplied the amount of nitrogen found in the milk, it is obvious that these positive balances were attributable to the maintenance ration. It appears therefore that, for animals in a resting stage—particularly when such animals are employed in accurate nitrogen metabolism studies—the maintenance ration should be reduced from the normal standard of 0·6 lb. protein per 1000 lb. live weight. Otherwise there is a definite risk that the maintenance ration will either contribute to milk production or will result in positive nitrogen balances, both of which would tend to vitiate the calculation of the biological values of the food proteins.

Special attention must be drawn to the figures for the cow Milkmaid. At the commencement of the meat-meal period an accident occurred through which she became lame, and a large oedematous swelling formed on her side. It is probable that the resulting temporary interference with normal metabolism was responsible for the high faecal N (Table VII) and the negative nitrogen balance (Table VIII) of Milkmaid during this period.

(*g*) *Calculation of biological values.*

In the past few years the idea that each protein mixture possessed a definite nutritive value has gained currency. Thomas gave the name "biological value" to this figure and defined it as the percentage of absorbed nitrogen which could be used for anabolic purposes. Up to the present time most of the work on biological values has been concerned with growth and maintenance. Relatively few investigations have been made into the biological

Table VIII. *Nitrogen balances.*

Cow	Period	Intake g. N	Output g. N	Gain or loss for period g. N	Daily gain or loss g. N
Mona	Beans	2989·77	2843·28	+146·49	+10·5
	Linseed	3004·33	2788·77	+215·57	+15·4
	Meat meal	2976·44	2620·94	+355·50	+25·4
	Beans	2989·77	2798·58	+191·19	+13·7
Butterfly	Linseed	2722·28	2526·07	+196·21	+14·0
	Beans	2725·69	2588·62	+136·69	+ 9·8
	Meat meal	2701·32	2497·42	+203·90	+14·6
	Linseed	2722·28	2554·24	+168·04	+12·0
Kate	Beans	2704·68	2634·49	+ 70·19	+ 5·0
	Linseed cake	2715·39	2735·63	− 20·24	− 1·4
	Meat meal	2711·85	2685·05	+ 26·80	+ 1·9
	Beans	2704·68	2525·56	+179·12	+12·8
Milkmaid	Linseed cake	2621·46	2521·42	+100·04	+ 7·1
	Beans	2630·75	2510·60	+120·15	+ 8·5
	Meat meal	2625·92	2763·31	−137·39	− 9·8
	Linseed cake	2621·46	2563·94	+ 57·52	+ 4·1

values for milk production. Hart and Humphrey (10), in an extensive series of experiments on both young and adult cows, obtained very variable results for the same protein. Two obvious fallacies are apparent in both this work and in the later experiments of Maynard *et al.* (22) and Holdaway *et al.* (23). Where young beasts were employed the ingested protein was used for both milk production and growth. With the adult animals the amount of protein fed was in excess of the actual requirements, and no allowance was made in the final calculation for the normal decrease in milk yield as lactation advanced.

It is clear that the biological value of any given protein mixture for milk production is the percentage of nitrogen absorbed from the production intake which is transformed into milk N, that is

$$\text{Biological value} = \frac{\text{milk N}}{\text{assimilated production N}} \times 100.$$

Assuming that none of the maintenance N intake is utilised for production, this may be written

$$\text{Biological value} = \frac{\text{milk N}}{\text{total assimilated N} - \text{assimilated maintenance N}} \times 100.$$

In the present experiment this assumption is justifiable, since the maintenance protein contained only minimal amounts of essential amino acids, and since slight positive N balances were obtained throughout.

Since total assimilated N = total N intake − (total faecal N − total metabolic N), and assimilated maintenance N = maintenance N intake − (maintenance faecal N − maintenance metabolic N), then

$$\text{Biological value} = \frac{X}{(I_t - (F_t - M_t)) - (I_m - (F_m - M_m))} \times 100,$$

$$= \frac{X}{I_t - I_m - ((F_t - M_t) - (F_m - M_m))} \times 100,$$

$$= \frac{X}{I - (F_t - M_t) + (F_m - M_m)} \times 100 \qquad \ldots(1),$$

where X = total milk N, I_t = total N intake, I_m = maintenance N intake, F_t = total faecal N, F_m = maintenance faecal N, M_t = total metabolic N, M_m = maintenance metabolic N, and $I = I_t - I_m$ = production N intake.

From this formula it is evident that the urinary N, either exogenous or endogenous, does not enter into the calculation of biological values for milk production. Therefore all nitrogen absorbed which is not converted to milk N is assumed to be wastage. It has already been pointed out that the positive N balances, which indicate protein storage in the body, can be attributed solely to the maintenance ration, and can therefore be neglected in the present calculations.

Estimation of the faecal metabolic N is essential before the biological value can be calculated. Thomas, and Martin and Robison assumed that this figure could be obtained by allowing the animal to eat a N-free diet, that is, that the metabolic N excretion is a constant figure. Mitchell (9), however, using rats, has shown that the roughage content of the diet, together with the total amount of dry matter present, could alter the figure. With cows it is almost impossible to arrange a diet containing the requisite amount of fibre and no nitrogen, and even if one could be found, it is almost certain that the animal would not eat it for the requisite periods. Morgen *et al.* (24) used a N-free ration consisting of extracted straw, starch, sugar, oil and minerals, and found that, for sheep, the average excretion of metabolic N in the faeces was 0·51 g. per 100 g. dry matter consumed. In the present investigation the only diet which an animal could be persuaded to eat was one of sago-pith meal, which contains only minimal amounts of nitrogen, but which is low in fibre. A quantity of this meal was therefore fed to a $2\frac{1}{2}$ year old steer, the amount fed being sufficient to cover the energy requirements of the animal. Table IX gives the results of the last 4 days' feeding, after a preliminary period of

Table IX. *Metabolic nitrogen of faeces.*

Animal	Day of feed	Weight of feed kg.	Output of faeces kg.	N in faeces g.	Average g.	Metabolic N as % of dry matter intake
Steer, 2½ years, 756 lb. live weight	4	7·5	16·75	28·30	28·86	0·405
	5	7·5	11·25	26·56		
	6	7·5	12·00	30·27		
	7	7·5	17·25	30·35		

3 days. The figure obtained (0·405 g. N per 100 g. dry matter intake) is lower than that found by Morgen *et al.* (24), and more nearly approaches that obtained for other animals (9).

It was also essential, before calculations of the biological values could be made, to obtain a figure for the faecal N attributable to the maintenance ration (F_m). Table X shows the amount of faecal N excreted when the maintenance ration of straw, oats and beet pulp (in the same ratio as was given

Table X. *Faecal nitrogen on maintenance ration.*

Animal	Weight of feed	Day of feed	Output of faeces kg.	N in faeces g.	Average g.	Faecal N as % of N intake
Steer, 2½ years, 756 lb. live weight	3·414 kg. straw	4	12·54	34·99	35·60	40
	1·000 kg. beet pulp	5	10·37	36·32		
	1·134 kg. oats daily	6	11·10	35·50		

to the experimental cows) was fed to the steer. The percentage of the total intake of nitrogen found in the faeces (40 per cent.) agrees very well with the calculated digestibilities of the complete ration.

Using these figures, the biological values of the protein mixtures were calculated according to formula (1). The results are shown in the right-hand column of Table XI. It will be seen that large variations occur with the same feed, and it is also apparent that there is a general tendency for the values to fall throughout each period. This is undoubtedly due to the fact that, while there is a normal fall in milk yield throughout lactation, the level of feeding has been kept constant for all periods, so that protein is being fed in excess of requirements in the later periods. This will tend to depress the average biological values of each of the feeds, and more especially of the meat meal, which has invariably been fed only in the third period. The mean values for each protein mixture (omitting Milkmaid, whose third and fourth periods were abnormal) actually work out as follows: bean-meal ration 52, meat-meal ration 44, linseed ration 42.

An attempt has been made to correct this overfeeding by the following method. The normal milk yield which would be anticipated *independent of feed changes* has been calculated from Figs. 2–5 for each of the experimental periods, and the production N intake ($I_{(corr.)}$) has been based on this calculated milk yield. That is to say, the production N intake has been decreased for each succeeding period. Concurrently a correction has been made for the faecal N (F_x) and the metabolic N (M_x) due to the excess production N intake

Table XI. *Biological values (calculated according to formula (2)).*

Cow	Period	% decrease in milk N from first period	X	$I_{(corr.)}$	F_t	M_t	F_m	M_m	F_x	M_x	Biological value, by formula (2)	Biological value, by formula (1)
Mona	Beans	—	72·25	148	92·72	59·27	39·34	29·84	—	—	58	58
	Linseed	7·6	57·07	136·75	74·65	53·70	39·34	29·84	2·25	1·80	46	41
	Meat meal	16·2	55·90	124	89·38	56·50	39·34	29·84	4·80	4·00	55	45
	Beans	22·7	60·20	114·4	86·76	59·27	39·34	29·84	6·72	6·00	62	46
Butterfly	Linseed	—	74·04	134	66·67	53·70	39·34	29·84	—	—	56	56
	Beans	8·1	72·00	123·15	77·99	59·27	39·34	29·84	2·17	2·16	63	58
	Meat meal	16·9	66·80	111·35	72·06	56·50	39·34	29·84	4·53	4·05	63	52
	Linseed	26·4	40·68	98·62	63·67	53·70	39·34	29·84	7·08	6·18	41	30
Kate	Beans	—	71·80	137	70·21	50·87	33·63	25·46	—	—	57	57
	Linseed cake	5·9	54·52	128·92	60·68	47·60	33·63	25·46	1·62	1·20	44	41
	Meat meal	12·6	44·79	119·74	83·99	49·50	33·63	25·46	3·45	2·81	48	40
	Beans	19·4	47·72	110·4	77·82	50·87	33·63	25·46	5·52	4·15	51	40
Milkmaid	Linseed cake	—	72·41	131·2	67·90	48·24	33·63	25·46	—	—	61	61
	Beans	7·7	67·15	121·1	81·30	47·47	33·63	25·46	2·02	1·80	70	63
	Meat meal	16·8	57·75	109·16	106·93	47·40	33·63	25·46	4·41	3·65	99*	112
	Linseed cake	24·4	63·71	99·19	65·60	48·24	33·63	25·46	6·40	5·44	70*	52

* Omitted, due to accident and abnormal metabolism.

which was actually fed, the faecal N being calculated from published digestibility values. In this way we arrive at formula (2):

$$\text{Biological value} = \frac{X}{I_{(\text{corr.})} - (F_t - M_t) + (F_m - M_m) + (F_x - M_x)} \times 100 \quad \ldots(2).$$

Clearly this formula can only be considered approximate, since three of the figures are obtained from interpolated data. Table XI shows the biological values of the rations for each feeding period calculated according to this formula. The mean values obtained with all four animals (excepting Milkmaid's third and fourth periods) demonstrate that the bean-meal ration is superior for milk production to both the linseed and meat-meal rations, the value for the meat-meal ration falling midway between the bean-meal and the linseed rations.

It is true that there are considerable variations for the same feed. If, however, reference is made to Table XII, in which the mean values are tabulated, it will be seen that the relative values obtained with each individual animal show a marked consistency.

Table XII. *Mean biological values calculated by formula* (2).

Ration	Mona	Butterfly	Kate	Milkmaid	Mean, omitting Milkmaid
Bean meal	60	63	54	70	59
Meat meal	55	63	48	—	55
Linseed	46	48	44	61	46

It must be clearly understood that the biological values noted in this paper refer, not to the pure foods (beans, linseed or meat meal), but to these foods supplemented by oats. Further, while the production ration was fed in correct quantities, it is probable that the maintenance ration was fed at too high a level, as indicated by the positive nitrogen retentions in 14 out of the 16 feeding periods. The possibility of a supplementary relationship between the proteins of the maintenance ration and the production ration must also be taken into account. With these reservations, it may be concluded that, when fed under the conditions noted in this paper, beans provide a better source of nitrogen for milk production than meat meal or linseed, the relative values of the mixed rations containing these concentrates being: bean-meal ration 59, meat-meal ration 55, linseed ration 46.

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57. A BACTERIAL MILK TAINT

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The literature on the microbiology of milk contains numerous references to the activities of micro-organisms producing taints. In such cases, while there is frequently a considerable volume of evidence associating particular bacteria or other organisms with the production of the characteristic changes described, much less information is available as to the sources of the causal organisms concerned. One of the greatest difficulties encountered in the investigation of taints is due to the fact that they are not infrequently of an intermittent nature and may, therefore, disappear before detailed information regarding them can be secured. In the case under consideration the taint was of a sufficiently permanent nature to allow the source of the causal organisms to be determined, and it appears advisable, therefore, to place on record a description of the observations which have been made.

During the spring months of 1929 a milk producer reported that he had received complaints that the milk supplied by him often developed an objectionable "heavy" odour. This producer sent milk to a local creamery and he stated that the taint was frequently so pronounced, and developed so rapidly, that the milk was rendered unfit for use before it could be pasteurised. In certain cases the taint had been observed to appear as early as 6–8 hours after milking, and affected churns always contained milk from the morning milking.

The general level of hygiene maintained in the production and handling of the milk in question was above the average for the district. In 1927 the dairy concerned had taken part in a clean milk competition held in the county. In this the staff, which in the interval had remained unchanged, acquitted itself creditably. Samples of milk submitted for examination in connection with the competition showed a bacterial content which did not exceed 12,000 per c.c., and coliform organisms were absent from 1/10 c.c. in all of the samples examined. The buildings and equipment were of an up-to-date character, and in most respects their hygienic condition was satisfactory. It was observed that, although the floor of the cowshed had a cement surface, the standings were not regularly washed owing to the risk of the cows slipping. It was also ascertained that the morning milk was dispatched uncooled, while the evening milk was passed over a water cooler and forwarded to the creamery next day.

Samples of the tainted milk, when received in the laboratory for examination, were normal in appearance, flavour and aroma. Microscopic examination of stained films from the centrifuge sediment showed few bacteria which

appeared to be of the types usually found in milk. The bacterial content of the fresh samples, as determined by plating on glucose agar, was not abnormally high, and there were no indications of excessive faecal contamination. Within 24 hours, samples of the milk kept at 22° C. generally developed a characteristic, penetrating odour with a tendency to cause irritation to the throat. The aroma closely resembled that of amyl alcohol and the flavour was slightly oily. The characteristic aroma appeared, though less rapidly, in milk kept at room temperature (10–15° C.). It was also observed in samples incubated at 30 and 37° C., but at these temperatures it could not be detected in all samples in which it developed at lower temperatures. Stained films prepared from tainted samples showed groups of micrococci.

The fact that the characteristic aroma was absent from fresh samples but appeared in samples which had been kept for some time was in harmony with the view that the causal agent was of a microbiological nature. This theory received further support from the results of a series of inoculation experiments carried out with samples of tainted milk. Duplicate samples of fresh certified and of sterilised milk were inoculated with small quantities (approximately 2 per cent.) of affected samples and kept at temperatures ranging from room temperature to 37° C. After an incubation period depending upon the temperature, the aroma of amyl alcohol could be detected in all of these samples. Uninoculated control samples of the certified milk employed, when kept under the same conditions as those which had been inoculated, failed to show any trace of the taint.

The causal organisms.

A search was next made for an organism capable of producing the taint. Samples of milk which had already developed the characteristic aroma were plated aerobically on glucose agar at 22 and 37° C. After an incubation period of 1–2 days a pronounced smell of amyl alcohol could be detected in the Petri dish cultures, which showed mainly colonies of micrococci. In some samples these organisms appeared to be in almost pure culture. Representative colonies of the various types present were purified by plating on glucose agar and tested with a view to determining their ability to produce the characteristic aroma. Of the cultures examined, only the micrococci were capable of producing the taint when transferred to tubes of sterilised or certified milk.

The strains of micrococci studied were of two main types. The majority (nine in number) produced whitish growths on most media. In addition to these, however, three strains of orange micrococci were obtained.

The characteristics of the white strains were as follows:

Morphology. The individual cells were medium to large cocci, generally arranged in pairs or irregular groups. They were rarely quite spherical but usually appeared somewhat flattened. The size of the cocci varied greatly, but their maximum breadth was generally about 1·5μ when the cells were measured in living preparations. Endospores were not observed.

Motility was absent.

Staining. The cells were stained well and uniformly by dilute fuchsin. They were positive when treated by the Kopeloff and Beerman and the Hucker modifications of the Gram technique.

Cultural characteristics.

The cultures grew at 22 and 37° C. They were facultative anaerobes, but growth under anaerobic conditions was scanty. Some of the strains were capable of growing in peptone water at *p*H 5·4, while others failed to do so. All grew at *p*H 8·0, which was the highest value tested.

Ordinary agar plate. Surface colonies were white, opaque and round with a smooth glistening surface. The edges of the colonies were uniform or very slightly irregular. Deep colonies were opaque and spherical, ovoid, lenticular or irregular. Microscopically both surface and deep colonies were very dense and compact but the surface colonies appeared finely granular, especially near the edge.

Ordinary agar slope. The growth was white, opaque and glistening with a uniform or slightly wavy edge. It was of medium thickness.

Gelatine plate. Surface colonies were similar to those on agar. Deep colonies were irregularly spherical. Liquefaction was comparatively rapid: it began in about 4 days and was generally complete in about one week. When examined microscopically the liquefying colonies showed a very irregular edge with coarsely granular marginal zone, and in some of the surface colonies there was a translucent ring with markings of a similar nature extending from it towards the centre. The colonies showed a tendency to remain intact in the liquid gelatine which otherwise appeared clear.

Gelatine stab. The stab was at first thread-like, but after 2–4 days an elongated narrow funnel-shaped zone of liquefaction appeared. Liquefaction then proceeded slowly, until at the end of a month it was almost complete. The liquefied gelatine rapidly cleared and some flocculent greyish sediment was deposited.

Bouillon. After 2–3 days at 22° C. a uniform turbidity of moderate density was produced with, as a rule, a thin discontinuous greyish scum. The liquid then gradually cleared and a granular deposit accumulated at the base of the tube. The reaction became distinctly alkaline to phenol red.

Glucose agar stab. The surface growth was at first greyish white, slightly raised, opaque and glistening with a uniform edge. Later the edge became lobate and some radial internal markings appeared, but the growth did not extend to the sides of the tube. The stab was thread-like to slightly papillate. No gas was produced.

Milk. A soft curd was formed in 1–2 days at 37° C. and in 2–6 days at 22° C., and almost immediately after coagulation a small quantity of clear liquid appeared on the surface of the curd. The latter then gradually diminished in volume and was replaced by a whitish turbid fluid. After 2–4 weeks, only

a small quantity of residual curd remained at the base of the tube. When curdling took place the medium was acid to brom-cresol purple and the reaction remained acid even after an incubation period of 1 month.

Potato. For about 10–14 days at 22° C. there was no visible growth: then a few small, round, raised colonies of the same colour as the medium appeared.

Biochemical reactions.

Acid was formed in peptone water containing dextrose, levulose, galactose, saccharose and lactose and nitrate was reduced to nitrite. The indol, methyl red and Voges-Proskauer reactions were negative. The organisms failed to grow in O'Meara's fumarate and Simmon's citrate media, and they were incapable of utilising uric acid, asparagin, ammonium salts and nitrates.

In most of their characteristics the orange strains resembled the white forms. They differed from them, however, in the following respects:

(1) The surface colonies on agar ultimately became markedly lobate.

(2) The growth on gelatine, agar and potato was orange-coloured. On potato an abundant growth was produced after 2–3 days' incubation at 22° C. When the strains were first isolated their growth on agar was of a bright orange colour. After some time, however, the colour became paler and ultimately the organisms produced a pale cream-coloured growth on this medium. In the course of a series of replatings of certain strains, after these had been in pure culture for more than 2 years, it was observed that the plates from one strain contained both orange and cream-coloured colonies. Representative colonies of both types were replated several times and characterised. The orange and cream-coloured cultures thus obtained were identical in all respects except colour, and both produced the characteristic aroma of amyl alcohol on suitable media.

The general characteristics of the white strains agree closely with those usually ascribed to *Micrococcus caseolyticus* Evans (2). According to Hucker (5) *M. casei liquefaciens* strain No. 9, in the collection maintained by Orla-Jensen, is a typical member of this species. A strain of this organism, kindly supplied by Prof. Orla-Jensen, was examined and compared with the white strains described above. This culture grew less abundantly than the white strains. Further, the changes produced by it in media, although essentially of the same type, were considerably delayed as compared with those due to growth of the white strains. These differences in cultural characteristics were, however, of a quantitative rather than of a qualitative nature and were such as might be attributed to the fact that *M. casei liquefaciens* produces a more meagre growth than the strains isolated in this investigation. With reference to the difference in the amount of growth formed, it may also be pointed out that Hucker states that micrococci of the *casei liquefaciens* type produce a medium (5) or even luxuriant (4) growth on solid media.

The orange strains differ from the majority of orange micrococci described

in the literature. Under the designation *M. caseolyticus* Miss Evans (2) included cultures which produced yellow to orange pigments. These strains were culturally identical in all essential characteristics with the white strains, which some later workers have regarded as typical of *M. caseolyticus*, except in the colour of the pigment produced. Although Miss Evans submitted no evidence other than similarity in cultural characteristics of the relationship of the coloured to the white forms, in view of recent work on the micrococci it appears not improbable that such a relationship does exist. Bigger, Boland and O'Meara (1) have recently recorded the transformation of *M. albus* to *M. aureus* and the reversion of *aureus* to *albus*, while Mellon and Caldwell (6) have published data which they consider support the view that these organisms have originated from a common ancestor. Pinner and Voldrich (11) observed the transformation of *aureus* to *albus* strains as well as the reversion of *albus* to *aureus*. It appears probable, therefore, that the orange strains of micrococci isolated from tainted milk should be classified with the corresponding white forms.

PRODUCTION OF THE TAINT.

Pure cultures of the micrococci studied showed considerable variation in their capacity to produce the characteristic aroma in sterilised milk. With active strains, the taint as a rule could be detected in 1–2 days at 22° C. and sometimes in less than 24 hours at higher temperatures. The white strains were generally more active than the coloured forms, but in cultures of *M. casei liquefaciens* No. 9 no trace of the taint could be detected. Once acquired, the ability to produce an aroma resembling that of amyl alcohol was retained by strains in pure culture for considerable periods. The micrococci isolated in these experiments have retained this property for a period of more than 3 years. In milk inoculated with strains of *Streptococcus lactis* or butter starters in addition to active strains of the micrococci, the development of the aroma was observed to be rather erratic. In certain cases it was quite as rapid as in sterile milk inoculated with the micrococci alone, while in other instances it appeared to be more or less completely suppressed.

Under aerobic conditions the smell of amyl alcohol was detected in all media in which growth took place. In certain cases, however, it appeared in liquid media before the latter showed a perceptible turbidity. On the other hand, although a slight growth took place under anaerobic conditions on media in which aerobically the aroma was produced, no trace of the latter could be detected in cultures which had been incubated in absence of oxygen.

It has already been observed that the micrococci isolated in this investigation were unable to utilise nitrate, ammonium salts or asparagin as their sole source of nitrogen. On media containing protein or peptone, however, good growth was obtained. The micrococci grew and produced the characteristic aroma in ordinary and skimmed milk, nutrient agar (both extract and infusion), nutrient gelatine, bouillon (extract and infusion) and peptone

water. The latter was a particularly good medium for the production of the aroma and in this respect Difco proteose-peptone appeared to be slightly superior to Difco bacto-peptone. The addition of 0·5 per cent. glucose to peptone water or to agar tended to retard the development of the aroma in these media, but the addition of 0·2 per cent. nitrate to nutrient agar was without effect upon this property. Attempts to separate from peptone water cultures a substance possessing the properties of amyl alcohol were unsuccessful.

Comparatively few references to the formation of amyl alcohol by microbiological agencies have been found in the literature. Several workers—Morin (7), Perdrix (9), Péreire und Guignard (10), Pringsheim (12), Schardinger (14)—have recorded the occurrence of the compound among the products of growth of the butyric acid bacilli. In these cases, however, amyl alcohol was as a rule accompanied by other characteristic substances such as butyric acid and gases, and it was the product of the activity of more or less strictly anaerobic bacilli. From the microbiological standpoint, therefore, these changes have little in common with those described in this paper.

Iso- and *d*-amyl alcohols are formed in small quantities in the alcoholic fermentation in which they constitute a large proportion of the so-called fusel oils. Ehrlich has shown that yeast is capable of converting leucine to *iso*-amyl alcohol and *iso*-leucine to *d*-amyl alcohol. His work is discussed by Harden (3) in whose monograph references to Ehrlich's papers will be found. Since the appearance of Ehrlich's publications Pringsheim (13) has demonstrated that a number of fungi—*Mucor racemosus*, *Rhizopus tonkinensis*, *Monilia candida* and a Torula—can also bring about the transformation of leucine to amyl alcohol, and Nawiasky (8) has submitted evidence that *B. proteus vulgaris* is capable of producing a similar change. It appeared, therefore, to be of interest to determine whether the micrococci described in this paper could produce the characteristic aroma in media containing leucine as the sole organic compound. In these experiments samples of *l*-leucine and *iso*-leucine supplied by Kahlbaum were employed. The compounds were dissolved separately in 0·05 per cent. potassium phosphate (K_2HPO_4) solution, the reaction of the solution was adjusted to *p*H 7·0 and the media so prepared were sterilised by filtration through Berkefeld N filters. New filter-candles, which had been washed by drawing water through them until the washings showed a constant reaction, were used. Strains of the micrococci described grew readily in leucine and *iso*-leucine media and in both they produced a marked aroma resembling that of amyl alcohol, while uninoculated control tubes of the leucine media, incubated with the inoculated cultures, showed no trace of the characteristic aroma. Media containing casein as the only source of nitrogen were also tested and found to be able to support the growth of the micrococci and to allow them to produce the smell of amyl alcohol. These results are in harmony with those of the experiments with leucine.

SOURCE OF THE CAUSAL ORGANISMS.

It has already been noted that the taint was generally observed in milk from the morning milking and that this was sent to the creamery uncooled. By thoroughly cooling the morning milk immediately after milking it was possible to delay considerably the development of the taint and as a result of this treatment the milk could, as a rule, be pasteurised before the aroma of amyl alcohol appeared. Nevertheless, it seemed advisable to attempt to trace the source of the causal organisms with a view to eliminating them altogether.

The taint was first observed during the winter and spring months. During this period the cows were housed continuously and, when samples of the mixed milk were kept for 1–2 days, they regularly developed the aroma of amyl alcohol. In the summer months, however, when the animals were continuously at grass except when they were being milked, the taint completely disappeared. When the cows were housed continuously in the subsequent autumn, the mixed milk again developed the taint.

During the winter months the characteristic aroma was observed not only in the mixed milk of the herd but also in that drawn into sterile bottles from individual cows. In order to determine whether the taint was confined to the milk of a few cows, the herd was divided into groups, each composed of seven or eight animals. From each group a composite sample was taken by milking into a sterile bottle, without special precautions, a small quantity of milk from each cow. When kept for a few days all the composite samples developed the taint, indicating that contamination was probably not confined to the milk of a few animals. The udders and flanks of four cows selected at random were thoroughly washed and disinfected with lysol and milk samples were drawn as aseptically as possible into sterile bottles. The taint did not develop in these samples. It therefore appeared improbable that the causal micrococci were present in the cows' udders.

The results of the experiments described in the last paragraph support the view that contamination was probably derived from the flanks, udders and teats of the cows. An examination was made of hairs obtained from the cows' udders and flanks and of a number of other possible outside sources of contamination, such as the litter, feeding stuffs and drinking water, and scrapings from the floor of the cowshed and from the cows' troughs. Small quantities of the various materials were transferred to sterile milk which was kept for some days at room temperature or until it developed the taint. It was soon discovered, however, that the presence of organisms capable of producing the characteristic aroma was more easily detected by plating quantities of the different materials on ordinary agar. Plate cultures inoculated with materials containing the micrococci produced an aroma of amyl alcohol after 2-3 days at 22° C. Of the various possible sources of contamination examined, only the cows' hair and scrapings from the floor of the cowshed yielded cultures

which showed the characteristic smell. From each of these sources organisms similar to both the white and orange strains of micrococci obtained from tainted milk samples were isolated.

Before steps could be taken to eliminate the causal micrococci it was necessary to ascertain whether the latter were being conveyed by the milk utensils. A number of milk pails, railway churns and other utensils ready for use were, therefore, washed internally with sterile water, which was then examined for the presence of micrococci capable of producing the taint. The results of these experiments yielded no support to the view that contamination was being conveyed by imperfectly sterilised utensils.

The evidence presented appears to justify the conclusion that the source of the causal organisms of this taint was the surface of the floor of the cowshed. From the floor the micrococci probably passed to the coats of the cows and thence into the milk at milking time. The fact that milk drawn without aseptic precautions developed the taint, while that obtained after the udders and flanks of the animals had been disinfected failed to do so, harmonises with this view. The latter also receives support from the disappearance of the taint when the cows were at grass. During this period the animals were only housed for milking purposes. Their coats, therefore, had little or no opportunity of coming into contact with the floor of the cowshed. An attempt was made to eliminate the taint completely by washing and disinfecting the cows' standings. The floors of the stalls were scraped, washed and thoroughly scrubbed with cresol. When this treatment was repeated at intervals the taint gradually disappeared from the milk even when the cows were continuously housed. This observation furnishes additional evidence in support of the view that the source of the causal organisms was the floor of the cowshed.

Summary.

1. The production in milk of an aroma resembling that of amyl alcohol was due to the growth of white and orange micrococci, similar to those described as *M. caseolyticus* by Evans (2).

2. The taint was observed when the cows were housed, but disappeared when they were put out to grass.

3. The source of the causal micrococci was found to be the floor of the cowshed. The taint disappeared when the latter was washed and disinfected.

4. The aroma of amyl alcohol was produced in media containing leucine as the sole organic compound.

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58. COLIFORM ORGANISMS IN MILK AND BOVINE FAECES

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In the bacteriological examination of raw milk in England it is customary to test for coliform organisms and to regard the extent to which they occur as an indication of the care with which the milk is produced and subsequently handled.

The present test is presumptive, and the presence of acid and gas in a bile salt lactose broth, containing litmus, Andrade's or other indicator is considered to denote the presence of coliform organisms. These organisms do not ordinarily occur in the udder of the cow, and under ideal conditions should not be present in milk. In practice it is rarely possible to exclude them completely, but in clean milk, handled and distributed under good conditions, their numbers, as estimated by the presumptive test, are very small, even when the milk is 24 hours old (Mattick and Stenhouse Williams (1)). In view of the fact that the presence of coliform organisms is also frequently interpreted as an indication of faecal contamination, it was felt that it would be of interest to obtain further information, using modern technique, on the types of facultatively anaerobic lactose-fermenting, non-gelatin-liquefying, Gram-negative bacteria occurring in milk and in bovine faeces.

Rogers, Clark and Davis (2) divided the Gram-negative lactose-fermenting bacteria into two groups, those giving a high, and those giving a low ratio of $CO_2 : H_2$ in a dextrose medium, and found the two groups to be present in almost equal proportions in milk. Rogers, Clark and Evans (3) found that from bovine faeces 149 out of 150 belonged to the low ratio group, but that from grain (Rogers, Clark and Evans (4)) 151 out of 166 cultures were of the high ratio type, which they recognised as *Bact. aerogenes*; similar lactose-fermenting, Gram-negative organisms, which, however, slowly liquefied gelatin, were regarded as *Bact. cloacae*.

Following the work of Clark and Lubs (5); Levine (6), Rogers, Clark and Lubs (7), and Koser (8) showed that the methyl red, Voges-Proskauer, indol and citric acid utilisation tests were correlated and these tests are now used to differentiate the faecal from the non-faecal coliform organisms.

Koser (8) found that the citrate test gave more reliable information regarding the source of the organism under test than the methyl red and Voges-Proskauer tests. Chalmers (9) working on coliform organisms in milk used the classification of Stewart, and included many non-lactose-fermenting, Gram-negative, non-spore-forming organisms.

In the present investigation the term coliform organism has been restricted to Gram-negative, non-spore-forming facultatively anaerobic organisms, fermenting lactose in the presence of bile salt, with the production of acid and gas. Milk and bovine faeces have been examined, and the relative numbers of *Bact. coli* and *Bact. aerogenes* occurring amongst the colonies isolated from each have been determined.

Preliminary isolations.

Lactose bile salt tubes showing acid and gas were obtained from routine examinations of clean and dirty milk. Tubes of lemco broth were inoculated with one loopful of the material and incubated at 37° C. overnight. The broth cultures were streaked on MacConkey's agar plates and incubated for 48 hours at 37° C. From each plate portions of five acid-producing colonies, fished at random, were stained by Gram's method and examined. Part of each colony found to be Gram-negative was streaked on an agar slope, and part inoculated into lactose bile salt medium. Those cultures giving acid and gas were further examined to determine their type.

The usual tests with slight modifications were used to differentiate *Bact. coli* from *Bact. aerogenes*.

Methyl red test.

The medium for this test consisted of glucose (0·5 per cent.), peptone (1·0 per cent.) and water.

It was found that the examination was best made after 5 days' incubation at 37° C., since tests showed that some strains of *Bact. aerogenes*, if examined earlier, gave a positive reaction, *i.e.* the reversal to the negative reaction did not occur until the fifth day.

Indol test.

The medium used consisted of 1 per cent. peptone with 0·5 per cent. NaCl. This test was also carried out after 5 days' incubation, it being found that reactions which were indefinite earlier, became positive after this time. Ehrlich's reagent was used.

Voges-Proskauer test.

O'Meara's modification (10) involving the addition of creatin was used and found to be excellent, but instead of the Koser citrate medium plus glucose (0·5 per cent.) used by O'Meara, it was found that good results could be secured by using the simple medium described for methyl red tests.

The tests were made after 2–5 days' incubation at 37° C. when the results were found to be clear cut.

Citric acid fermentation test.

The method of Koser (11) was used for this test, the results being read after 2–3 days' incubation at 37° C.

Gelatin liquefaction.

A medium containing 20 per cent. gelatin and 1 per cent. peptone was inoculated with the test organism and examined for liquefaction at intervals during the incubation, for 28 days at 22° C.

Many of the cultures isolated from milk, when examined microscopically, showed bloated and irregular forms, many of which were capsulated, whilst the growth on agar was often mucoid.

Of 55 colonies isolated on MacConkey's agar, as described in the preliminary experiments, only very few gave the reactions either of *Bact. coli* or of *Bact. aerogenes*. The results are given in Table I.

Table I.

No. of cultures	Koser's citrate	Methyl red	Voges-Proskauer	Indol	Gelatin	Classification
14	−	+	−	+	−	*Bact. coli*
9	+	−	+	−	−	*Bact. aerogenes*
2	+	−	+	−	+	*Bact. cloacae*
7	+	+	+	+	−	Indefinite forms
12	+	+	+	−	−	
6	+	+	−	+	−	
3	+	+	−	−	−	
2	+	−	+	+	−	

It will be seen that of a total of 55 colonies examined, no less than 30 gave reactions which were indefinite, due probably either to mixed colonies or to the so-called "intermediate" forms. An attempt was therefore made to resolve these indefinite forms by using media claimed to differentiate *Bact. coli* from *Bact. aerogenes*.

Tests of differential media.

The media used were:

(1) Levine's eosin methylene blue agar (12).
(2) Chalmer's modification of MacConkey's agar (9).
(3) "Endo's" medium (13).
(4) Tonney and Noble's ferro-cyanide citrate agar (14).

Working at first with pure cultures of *Bact. aerogenes* (national collection of type cultures) and of *Bact. coli* (laboratory stock cultures) giving the recognised reactions, by streaking and by the pour plate method using a number of different proportions of the organisms, it was found that, in the hands of the writer, at any rate, none of the media tried gave clear cut differentiation between the two types of organism.

When the organisms taken from milk were tested on the same media indefinite reactions which did not conform to either type were still found. If the assumption that the cultures were mixed was correct it appeared that none of the media used was capable of resolving them, and the following medium was devised.

Koser's (11) citrate solution was solidified with 1·5 per cent. agar and varying

concentrations of crystal violet or brilliant green added. After a number of trials it was found that the best results were secured with a medium of the composition:

NaCl 0·25 per cent., sodium citrate 0·1 per cent., $MgSO_4$ 0·01 per cent., $(NH_4)H_2PO_4$ 0·05 per cent., K_2HPO_4 0·05 per cent., agar 1·5 per cent., brilliant green (1/2500) 1 per cent.

Known pure cultures of *Bact. coli* and *Bact. aerogenes* were streaked on this medium both separately and mixed in varying proportions. After 48 hours' incubation at 37° C. the plates of *Bact. coli* showed no growth, but *Bact. aerogenes* gave growth sufficiently good to allow of picking colonies.

Plates made from the mixed cultures also gave good growth and colonies picked off proved to be *Bact. aerogenes* unmixed with *Bact. coli*.

When the solution of brilliant green was raised to a concentration of 1/1000 and added to the medium at the rate of 1 per cent., *Bact. aerogenes* grew only slowly and *Bact. coli* not at all. With a less concentrated solution (1/5000) *Bact. aerogenes* grew well, but colonies of *Bact. coli* appeared after 2 or 3 days' incubation. Even with the higher concentration (1/2500) of brilliant green, colonies of *Bact. coli* appeared after 5–6 days when mixed cultures were used. It is possible that this was due to the breakdown of the citrate by *Bact. aerogenes* to carbon compounds available to *Bact. coli*.

Nevertheless it was found that on testing the colonies which had formed after 48 hours' incubation on the medium containing the 1/2500 solution of brilliant green, only *Bact. aerogenes* could be demonstrated.

The indefinite (or mixed) cultures isolated from milk when tried on this medium were also found after 48 hours' incubation to yield colonies of *Bact. aerogenes* only.

The predominance of *Bact. aerogenes* in this medium led to further experiments on the test media listed earlier, with pure cultures of *Bact. coli* and *Bact. aerogenes* mixed in varying proportions.

The results appear in Table II.

It seems clear from these experiments that if mixtures of the two organisms were sown in a citrate medium, *Bact. aerogenes* grew, even when heavily outnumbered by *Bact. coli*. In the case of the methyl red and indol tests, however, the reactions were generally those of *Bact. coli* and it will be seen that even when *Bact. aerogenes* was present in excess, the Voges-Proskauer reaction was usually negative, but on occasions was doubtful or even positive.

It appeared, therefore, that the original milk organisms giving irregular reactions on the test media were probably mixed cultures and not intermediate forms. From the 55 cultures (Table I) 110 pure cultures were secured by repeated streaking of broth cultures.

Two colonies were taken from each final plate, and the reactions of the 110 colonies obtained are given in Table III.

It is seen from this that the number of indefinites has been reduced from 30 to 6. These, in spite of repeated attempts to purify them further, have

remained true to type and must therefore be regarded as intermediate forms. Koser considers such methyl red positive citrate positive forms as soil organisms, and it would seem from these results that the numbers of soil organisms were slightly higher than would be suspected from the first results (Table I).

At this point attention must be drawn to the fact that the organisms whose reactions have so far been described had been taken from lactose bile salt tubes. It is possible, in view of the claims of Ruchhoft, Kallas, Chinn and

Table II.

Experiment Proportion of *Bact. coli* to *Bact. aerogenes*	Koser's citrate						Methyl red					
	1	2	3	4	5	6	1	2	3	4	5	6
Bact. coli	−	−	−	−	−	−	+	+	+	+	+	+
Bact. coli 3 / *Bact. aerogenes* 1	+	+	+	+	+	+	+	+	+	+	+	+
Bact. coli 1 / *Bact. aerogenes* 1	+	+	+	+	+	+	?	+	+	*N*	*N*	*N*
Bact. aerogenes 3 / *Bact. coli* 1	+	+	+	+	+	+	*N*	+	+	−	*N*	−
Bact. aerogenes	+	+	+	+	+	+	−	−	−	−	−	−

Experiment Proportion of *Bact. coli* to *Bact. aerogenes*	Indol						Voges-Proskauer					
	1	2	3	4	5	6	1	2	3	4	5	6
Bact. coli	+	+	+	+	+	+	−	−	−	−	−	−
Bact. coli 3 / *Bact. aerogenes* 1	+	+	+	+	+	+	−	−	−	−	−	−
Bact. coli 1 / *Bact. aerogenes* 1	+	+	+	+	+	+	−	−	−	+	?	−
Bact. aerogenes 3 / *Bact. coli* 1	+	+	+	+	+	+	−	−	−	+	?	?
Bact. aerogenes	−	−	−	−	−	−	+	+	+	+	+	+

N = neutral.

Table III.

No. of cultures	Koser's citrate	Methyl red	Voges-Proskauer	Indol	Gelatin	Classification
58	−	+	−	+	−	*Bact. coli*
42	+	−	+	−	−	*Bact. aerogenes*
4	+	−	+	−	+	*Bact. cloacae*
6	+	+	−	−	−	Intermediates

Total 110

Coulter (15), that owing to the presence of bile salt in the medium an incorrect picture of the relative numbers of *Bact. coli* and *Bact. aerogenes* in the original milk might have been obtained, due to the predominance of either organism in the presence of a deterrent such as bile salt.

Isolations directly from milk.

In view of the above possibilities a large number of samples of milk was plated, without preliminary enrichment in lactose bile salt tubes, on MacConkey's agar. From each MacConkey plate showing acid-producing colonies, five colonies were fished by random selection, stained by Gram and

examined. Many of the colonies consisted of mixtures of cocci and coliforms and had to be purified. All Gram-negative organisms giving acid and gas in lactose bile salt were then tested as before.

A large number of milk samples were dealt with and finally 37 cultures obtained. It should be noted that many milks were dealt with which failed to show the presence of coliforms.

Table IV.

No. of cultures	Koser's citrate	Methyl red	Voges-Proskauer	Indol	Gelatin	Classification
4	−	+	−	+	−	*Bact. coli*
13	+	−	+	−	−	*Bact. aerogenes*
6	+	+	−	−	−	Intermediate forms
2	+	+	−	+	−	
2	+	−	+	+	−	
4	+	−	+	−	+	
5	+	−	+	+	+	
1	+	+	−	+	+	

Table IV shows that of these 37 cultures only 4 were faecal *Bact. coli*, 13 being *Bact. aerogenes*, 10 "intermediates" and 10 gelatin liquefiers. The "intermediates" retained their characteristics in spite of repeated attempts to resolve them.

Although the results, by the nature of the experiments are not suitable for statistical examination there is a suggestion that direct plating of milk shows a lower relative number of faecal *Bact. coli* than when enrichment is resorted to, and tends to confirm the statements of Ruchhoft *et al.* (15). It is, of course, possible that the difference was due to the coliform distribution in the milk samples themselves, but it is certain that a large proportion of the coliforms obtained by direct plating of milk are of the soil type.

Coliform distribution in dung.

In view of the fact that numbers of *Bact. aerogenes* were present in milk it was felt that some experiments with dung would be of interest. For each experiment one loopful of fresh dung from a cow in a tubercle-free herd was inoculated into broth and incubated overnight at 37° C. Cultures were then made on MacConkey's agar and acid-producing colonies picked off and examined.

In general the coliform organisms from dung gave colonies which were less mucoid and were microscopically more defined in form, less pleomorphic and rarely capsulated.

The colonies were fished direct from MacConkey's plates and tested and without exception could be referred to one type or the other: no difficulty with mixed colonies was encountered.

Table V shows that of 47 colonies picked off, 44 were faecal *Bact. coli*, one was *Bact. aerogenes*, and two liquefied gelatin, but did not grow on citrate.

Table V.

No. of colonies	Koser's citrate	Methyl red	Voges-Proskauer	Indol	Gelatin	Classification
44	−	+	−	+	−	*Bact. coli*
1	+	−	+	−	−	*Bact. aerogenes*
2	−	+	−	+	+	?

It is clear from these figures that the proportion of *Bact. aerogenes* to *Bact. coli* in cow dung is small and very different from that obtaining in milk.

SUMMARY.

The lactose-fermenting coliform organisms in milk and dung have been investigated.

A large proportion of those recovered from raw milk by direct plating proved to be of the *Bact. aerogenes* type and a number of "intermediates" was also present.

In dung faecal *Bact. coli* largely predominated, the proportion of *Bact. aerogenes* being very small.

If the implication of these experiments be accepted it is possible that many of the coliform types found in milk are derived, not from faeces, in which the proportion of *aerogenes* is small, but from external sources, such as contaminated utensils, and from food-stuffs.

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59. STUDIES IN THE BACTERIOLOGY OF LOW-TEMPERATURE PASTEURISATION

PART I. THE SIGNIFICANCE OF HEAT-RESISTING ORGANISMS IN RAW MILK SUPPLIES

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In the course of routine bacteriological examinations of milk carried out at one of the United Dairies pasteurising depots during the past three years, a remarkable lack of constant relationship between the numbers of colonies reported on the 1/10, 1/100 and 1/1000 dilution plates, when standard agar (the Ministry of Health formula) was used, has been observed. When, however, standard agar, enriched with 0·5 per cent. milk, has been used as a plating medium, many of the discrepancies between different dilutions of the same milk have disappeared.

Fay (1), Mattick (2) and Hiscox (3) have reported the presence of bacterial colonies on the 1/100 dilution that are absent on the 1/1000 dilution when milk is plated with standard agar. In a recent paper by Hiscox *et al.* (4) attention is drawn to the fact that the quantity of milk added with the 1/100 dilution is not always sufficient to induce the growth of those bacteria for which standard agar alone is unsatisfactory.

Dotterrer (5) found that pasteurised milk gave more colonies on milk powder agar and by Frost's little plate method than on standard agar plates; he also failed to find any constant relationship between the bacterial content of raw milk and of the same milk when freshly pasteurised.

That the discrepancies in plate counts on standard agar are due largely to the lack of nutrient material is demonstrated in Table I, where the 1/100 dilution plate counts on standard agar and standard agar with the addition of 0·5 per cent. milk are compared. In every case recorded in this table the colonies developing on the milk agar plates proved to be streptococci.

Table I. *Bacteria per ml. in pasteurised milk.*

Standard agar	Milk agar
3,100	96,000
6,800	80,000
11,600	100,000
300	34,400
200	120,000
9,100	48,000
27,800	70,000
7,000	136,000
4,400	100,000

[1] This work did not come under my direction until May, 1932. E.B.A.

A further cause of discrepancy was found to be the occurrence of thermophilic organisms after a prolonged plant run.

Cooledge (6) has described a thermophilic bacillus showing active growth in slightly alkaline media. This organism was inhibited by the growth of acid-producing organisms, but when present in pure culture in sufficient numbers was capable of overcoming the slight acidity of standard agar.

The presence of thermophilic organisms in pasteurised milk, after a plant had been in use for some hours, has been reported by many observers, and a comprehensive review of the literature on this subject has been compiled by Breed (7). The organisms are rarely found in numbers exceeding 2000 per ml. in bulked raw milk supplies, but they are capable of rapid multiplication at pasteurisation temperature, and after a prolonged plant run have been found to be present to the extent of millions per ml.

Frequently, at the start of a pasteurising run when a bacteriological examination of the pasteurised milk was made, a far greater bacterial count was recorded on the 1/10 dilution plate than on the 1/100 dilution plate, on standard agar at 37° C. At the end of the plant run the position was reversed and the 1/100 dilution plate frequently recorded a far greater count than either the 1/10 or the 1/1000 dilution plates.

In Table II is summarised a series of tests made during the period April 1 to May 18, 1931. All plates were made on standard agar incubated at 37° C. for 48 hours.

Table II.

No. of tests	Pasteurised milk from	1/10 plate count greatly exceeded 1/100 plate count	1/100 plate count greatly exceeded 1/10 plate count	1/10 and 1/100 plate counts comparable	1/10 and 1/100 plate counts uncountable
111	Start of plant run	66	1	41	3
167	Finish of plant run	13	60	50	44

On examining the plates it was found that when the 1/10 dilution plate count exceeded the 1/100 dilution plate count the colonies present on the 1/10 were almost invariably streptococci. When the 1/100 plate count exceeded the 1/10 plate count the colonies on the 1/100 plate were almost invariably granular staining bacilli. On studying the two types of organism it was found that the streptococcus would grow only when a sufficient quantity of milk was present in the medium. The 1/100 dilution plate did not supply sufficient nutrient material for colony development.

When plated in pure culture the bacillus isolated from the 1/100 dilution plate would form colonies on both the 1/10 and 1/100 dilutions, but not on the 1/1000 plate. The organism failed to develop on the 1/10 plate when plated together with the streptococcus.

It would appear that the growth of the thermophilic bacillus is inhibited by the production of acid by the heat-resisting streptococci on the 1/10 dilution plate.

At the start of a plant run the thermophilic bacilli were either absent or present only in small numbers. At the end of a plant run, however, the thermophilic bacilli were present in greater numbers and, although they were inhibited on the 1/10 plate, the number of colonies which developed on the 1/100 plate was greater than one-tenth the number of thermoduric streptococcus colonies on the 1/10 plate.

The bacillus was also found to grow at 63° C. with good colony development on standard agar plates and in litmus milk with the production of slight acidity after 18 hours' incubation[1].

Robertson (8) has reviewed the literature on the thermal resistance of micro-organisms isolated from milk and has also, himself, isolated many cultures of heat-resisting organisms from milking machines that had been cleaned by means of a chemical sterilising agent.

Hucker (9), using fresh milk after 4 hours' storage at different temperatures, found wide variations in the suitability of different milk for pasteurisation. The predominating types of thermoduric streptococci isolated by Hucker were *Str. thermophilus*, *Str. faecium* and *Str. liquefaciens*. When milk had been held at 86° F. for 4 hours previous to pasteurisation the growth of *Str. thermophilus* was encouraged and plates made from such milk after laboratory pasteurisation contained a large proportion of this organism. It should be noted that Hucker used standard sugar-free media for his experimental platings, but he made no note of the dilution plate from which his cultures were isolated.

Sherman and Stark (10) have isolated heat-resistant strains of streptococci which they place in four groups:

(1) *Str. thermophilus.*
(2) *Str. bovis* including *Str. inulinaceus.*
(3) *Str. faecalis* including *Str. glycerinaceus.*
(4) *Str. liquefaciens* including *Str. zymogenes.*

All these strains were capable of resisting 145° F. for 30 min. in a 2-day culture at 98° F. Isolations were made from lactose agar plates.

Taylor (11), using the 1/100 dilution and standard agar as the plating medium, notes that a heat-resisting flora is the result of improper sterilisation of milk contact surfaces either on the farm or in the milk plant.

Hussong and Hammer (12) have recently published a report on the pasteurisation efficiencies secured with milk from individual farms. These workers failed to find any direct relationship between the bacterial counts of raw and laboratory pasteurised milk. They concluded that "more careful methods in the production and handling of the milk not only resulted in lower initial counts but also in higher pasteurisation efficiencies, so that the counts on the pasteurised milk were markedly decreased; when the initial counts were then increased, due to hot weather, the higher efficiencies and lower counts on the pasteurised product persisted."

[1] Recent experience has shown that the number of thermophilic organisms in pasteurised milk can be controlled by careful plant management, whereas the number of thermoduric organisms is mainly dependent on the bacterial flora of the raw milk supply.

In confirmation of the work reported by Hucker (9) it has been found that certain raw milk supplies show a far higher bacterial content after pasteurisation at 145° F. for 30 min. than others which originally contained a comparable number of organisms.

The present investigation was undertaken in order to determine the distribution and means of control of these heat-resisting organisms.

Experimental technique.

Laboratory pasteurisation of raw milk supplies was carried out in rubber-stoppered test-tubes. The milk was introduced into the test-tube by means of a 1 ml. pipette operated with a rubber teat, sufficient milk, about 10 ml., being introduced to cause the stoppered test-tube to sink when immersed in a water bath. Care was taken when transferring milk to the test-tubes to avoid any splashing up the sides of the tube.

When the milk was ready for pasteurisation the tightly stoppered tubes were completely immersed in a water bath at 145–146° F. Five minutes were allowed for the temperature of the milk to reach 145° F., the tubes were then maintained at that temperature for a further period of 30 min. The milk was cooled by immersing the tubes in cold water. Bacteriological examinations were made immediately before and immediately after laboratory pasteurisation.

The medium used throughout the investigation was standard agar of the composition recommended by the Ministry of Health with the addition of 0·5 per cent. sterile milk.

All plates were incubated at 37° C. for 48 hours.

Laboratory pasteurisation of milk from different sources.

In Tables III and IV are reported the bacterial counts of two types of bulk milk received at the processing depot.

Table III.

Bulk raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
880,000	800	99·9
1,560,000	340	99·8
1,480,000	140	99·9
5,000,000	90	99·9

Table IV.

Bulk raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
252,000	40,000	84·1
1,160,000	15,000	98·7
372,000	25,300	93·2
580,000	2,100	99·6
356,000	25,000	92·9
9,600,000	40,000	99·5

The milk tabulated in Table III was produced under exceptionally clean conditions—all utensils being steam sterilised twice daily. As there were no facilities for the refrigeration of these supplies during transport from the farm to the processing depot, the high bacterial content of the raw supplies was due to lack of refrigeration rather than any lack of care in the production of the milk.

The figures in Table IV are taken from ordinary milk supplies collected from the farm either once or twice daily, and refrigerated at a country depot before dispatch in three-thousand-gallon tanks to the town depot.

The high counts of these raw supplies are due to farm contamination rather than lack of refrigeration.

A comprehensive study of rail tank milk over a period of twelve months has shown that there are wide variations in the effect of pasteurisation when milk of different consignments is pasteurised in the laboratory. The results of this study are to be found in Table V.

Table V. *Laboratory pasteurisation of tank milk September* 1931 *to August* 1932.

Depot of origin	Number of tanks examined												
	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Total
A	23	15	4	19	14	36	18	17	28	24	27	33	258
B	12	6	2	6	4	6	2	6	9	7	11	12	83
C	18	13	3	13	6	17	6	4	7	19	48	16	170
D	4	13	5	7	—	8	2	—	7	3	3	7	59
E	8	6	2	4	3	3	3	2	5	—	1	—	37
F	10	—	1	2	1	—	—	3	13	17	16	9	72
G	—	—	—	—	—	1	1	2	7	15	14	—	40
	75	53	17	51	28	71	32	34	76	85	120	77	719

Depot of origin	Number of samples containing more than 30,000 organisms per ml. after laboratory pasteurisation													
	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Total	%
A	4	4	—	1	—	1	2	2	2	5	3	7	31	12
B	2	2	—	—	—	—	—	—	1	—	4	2	11	13
C	2	—	1	1	3	—	1	2	5	10	14	5	44	26
D	2	5	1	—	—	—	1	—	4	1	1	1	16	27
E	1	—	—	—	—	—	1	—	—	—	—	—	2	5·5
F	5	—	—	—	—	—	—	1	4	6	4	—	20	27
G	—	—	—	—	—	—	1	2	7	15	10	—	35	88
	16	11	2	2	3	1	6	7	23	37	36	15	159	21

It was found that in a total of 719 tank milks examined 159 (21 per cent.) contained more than 30,000 organisms per ml. immediately after laboratory pasteurisation. This figure bears no relation to the bacterial count of the raw milk supply.

The milk from depot G was collected twice daily. The bulk of the milk remained uncooled until its arrival at the country refrigerating depot. Many of the suppliers in this area had installed milking machines on their farms.

During September, 1931, when twice-daily collection of milk from depot F was in force, 50 per cent. of the samples contained excessive numbers of heat-resisting organisms.

Most of the examinations of milk from depot G were made during the months of May, June and July when 88 per cent. of the supplies contained more than 30,000 organisms per ml. after laboratory pasteurisation.

The comparative percentages for depots A, B and C for these 3 months are 12·5, 18·5 and 39 per cent. respectively.

Examination of milk from individual farms.

During the period June 20 to September 14 examinations were made of the milk from 104 farms where hand-milking was practised. The milk examined was usually a composite sample of mixed morning's and evening's milk delivered to the factory between 8 a.m. and 2 p.m.

The milk was examined bacteriologically by the plate-count method both before and after laboratory pasteurisation.

Of the 104 supplies 14 gave a count of over 30,000 organisms per ml. after laboratory pasteurisation. The results from some of the farms where high counts were obtained after laboratory pasteurisation are set out in Table VI.

Table VI. *Examination of hand-milked supplies yielding a high number of thermoduric organisms.*

Code	Raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
B 31	140,000	64,000	54·3
	620,000	54,000	91·3
	224,000	66,000	70·5
	1,060,000	172,000	83·7
B 19	204,000	14,000	93·1
	750,000	40,000	94·6
B 139	1,250,000	186,000	85·1
	2,400,000	30,000	98·7
B 470	226,000	73,000	67·7
	64,000	26,000	59·3
	179,000	102,000	43·0
B 278	200,000	111,000	44·5
B 243	350,000	91,000	74

In contrast to the figures in Table VI some results are reproduced in Table VII showing raw milk with an original high count where pasteurisation reduces the number of organisms to a far greater extent.

Table VII. *Examination of hand-milked supplies showing great reduction after laboratory pasteurisation.*

Code	Raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
B 2	500,000	30	More than 99·9
B 9	302,000	830	99·7
B 60	800,000	9100	98·8
B 76	560,000	910	99·8
B 89	400,000	1210	99·7
B 115	2,600,000	660	More than 99·9
B 194	370,000	4300	98·8
B 207	91,000	300	99·6

The milk obtained from fourteen suppliers who had installed milking machines was examined. The results are tabulated in Table VIII.

Table VIII.

Code	Raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
B 222	350,000	15,000	95·7
B 223	10,000,000	98,000	99·0
B 224	8,000,000	169,000	97·9
B 225	21,000	80	99·6
B 226	390,000	350,000	10·2
B 227	710,000	14,600	97·9
B 228	410,000	600	99·8
B 229	Millions	31,000	—
B 230	2,000,000	22,000	98·9
B 149	4,000,000	86,000	97·8
B 232	1,400,000	500	99·9
B 233	550,000	12,000	97·8
B 234	620,000	Less than 10	More than 99·9
B 242	330,000	114,000	65·4

The examination of milk from individual farms showed that 13 per cent. of hand-milked supplies and 43 per cent. of machine-milked supplies yielded bacterial counts exceeding 30,000 organisms per ml. after laboratory pasteurisation.

Though it is evident from Tables VI and VIII that only a proportion of the organisms present are of the heat-resisting type, their absolute numbers are often sufficient to interfere seriously with the efficiency of the pasteurisation process, with the result that the final product contains an unduly high number of living organisms.

Periodical examination of milk from individual farms.

In the majority of cases when periodical examinations were made the results were found to vary considerably. The explanation of this variation is probably to be found in the methods of utensil washing employed at the farm. In many cases the farm copper is only lighted at irregular intervals, the utensils being washed in cold or warm water on the other days.

In one instance heat-resisting organisms disappeared entirely from a supply that had previously contained large numbers. On the day before the test was made this farmer had a thorough "clean-up" of the dairy and all utensils before going away for a summer holiday.

The most striking case of milk regularly containing large numbers of heat-resisting organisms was that of farm B 226 where the milk was obtained by means of a mechanical milker.

Table IX records some typical results obtained from farms B 226 and B 278. This shows the daily variation which may be expected on many farms.

It is seen that there is practically no diminution of organisms as a result of pasteurisation of the milk from farm B 226, while the percentage reduction of organisms in the milk from farm B 278 varies considerably in different samples.

Table IX. *Laboratory pasteurisation of milk.*

Farm	Date	Raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
B 226	14. ix. 32	390,000	350,000	10·2
	23. ix. 32	230,000	218,000	9·2
	28. ix. 32	450,000	520,000	15·5 increase
	7. xi. 32	88,000	94,000	6·8 increase
B 278	28. ix. 32	200,000	111,000	44·5
	3. x. 32	49,000	770	98·4
	24. x. 32	58,000	4,900	91·5
	9. ii. 33	250,000	14,000	94·4
	10. ii. 33	119,000	18,800	84·2

The results from farm B 226 are of particular interest. The organisms present in the milk (almost a pure culture) appear to be identical with those obtained from the milk of depot G earlier in the year (see Table V).

Influence of cooling on the presence of heat-resisting organisms in milk.

Since the results obtained from depots F and G (Table V) indicated that non-cooling of milk at the farm might encourage the growth of heat-resisting organisms, individual supplies of cooled and uncooled evening's and morning's milk from certain farms were examined.

The results of these examinations are recorded in Table X, confirming the suggestion made earlier in the investigation and emphasising the importance of immediate cooling of milk on the farm.

Table X. *The influence of cooling on the presence of heat-resisting organisms in milk.*

Code	Milk	Raw milk Bacteria per ml.	Pasteurised milk Bacteria per ml.	% reduction as a result of pasteurisation
B 31	P.M. cooled	200,000	10,500	95
	P.M. uncooled	20,000,000	1,600,000	92
	A.M. cooled	240,000	19,300	92
	A.M. uncooled	138,000	19,700	86
B 226	P.M. uncooled	115,000,000	More than 30,000,000	—
	A.M. cooled	183,000	171,000	6
	A.M. uncooled	400,000	300,000	25
B 263	P.M. uncooled	10,000,000	30	More than 99·9
	A.M. cooled	6,000	20	99·6
	A.M. uncooled	29,000	10	99·9

Between 10 a.m. and 12 noon of the day of testing the morning's milk was delivered to the depot together with the previous evening's milk. The uncooled evening's milk from farms B 31 and B 226 when examined at the depot on the following morning contained excessive numbers of heat-resisting organisms, while the morning's uncooled milk with an interval of between 2 and 4 hours between milking and pasteurisation showed a much smaller number. When, however, uncooled morning's milk was kept for a period exceeding 4 hours after production there was a rapid increase in the number of heat-resisting organisms present (Table XI).

Table XI. *Pasteurisation of uncooled morning's milk from farm B* 226.

Age of milk hours	Temperature ° F.	Bacteria per ml. Raw milk	Bacteria per ml. Pasteurised
2	78	330,000	330,000
6½	68	680,000	1,500,000
8½	66	1,170,000	1,720,000
4½	73	710,000	1,050,000
7	65	760,000	3,150,000
8½	61	1,190,000	4,410,000
		Cooled control.	
2	—	168,000	166,000
6½	53·6	155,000	153,000
8½	53	186,000	182,000

Results from B 263 suggest that when heat-resisting organisms are absent from the raw supply the influence of non-cooling is not shown in the pasteurised milk.

The milk described in Table XI was delivered uncooled and remained in the depot, samples being withdrawn at intervals throughout the day. Not only was there an increase in the number of organisms in the raw milk, but there was a greater increase in the number of organisms after laboratory pasteurisation; in fact, the organism appears to be capable of growth at pasteurising temperature. The results are of the greatest importance because the organism found is one capable of souring pasteurised milk in less than 24 hours in hot weather.

SOURCE OF HEAT-RESISTING ORGANISMS ON THE FARM.

Frequent examinations of milk from farm B 226 showed that the milk contained almost a pure culture of heat-resisting streptococci.

On this farm a mechanical milker was in use. The farm routine for cleaning the milking machine and other utensils consisted of scrubbing all metal parts and immersing the buckets, cooler, strainers, etc., in boiling water. The rubber pipes and teat cups were first immersed in warm water, dipped into hot water from 140 to 160° F. for some minutes and finally left to soak in a cistern of cold water in the intervals between milkings. Serial examinations of the milk at this farm proved that the first point of contamination was the rubber milk tube.

When however, Ringer solution rinsings from the rubber tubes were pasteurised, no organisms survived. By replacing Ringer solution with sterile milk as a rinsing medium, many millions of heat-resisting streptococci survived laboratory pasteurisation. Similarly, when the water in which the rubber tubes and teat cups were soaked was pasteurised, no organisms survived, but when the water was mixed with an equal quantity of sterile milk before pasteurisation, it was found to contain many millions of heat-resisting streptococci.

The fact that the organism is killed by pasteurisation in a watery solution

but survives when heated in a milk medium has been confirmed by heating a 1/1000 Ringer solution dilution of heavily infected raw milk. The organisms present in the dilution do not survive pasteurisation, while the milk pasteurised undiluted shows no reduction as a result of heat treatment.

From investigations conducted at a number of farms it was apparent that the chief source of contamination of milk with heat-resisting organisms was in the milking utensils. In Table XII the influence of utensil sterilisation on the presence of these organisms is demonstrated.

Table XII. *The influence of utensil sterilisation on the presence of heat-resisting organisms in milk.*

	Before sterilisation Bacteria per ml.		After sterilisation Bacteria per ml.	
Farm	Raw	Pasteurised	Raw	Pasteurised
B 12	800,000	9,100	7,600	Less than 10
B 31	224,000	66,000	8,000	140
*B 93	4,000,000	139,000	1,280,000	Less than 100
B 139	1,250,000	186,000	15,000	Less than 10
B 523	32,000	18,100	7,000	300
B 527	550,000	200,000	1,300	Less than 100
B 530	450,000	120,000	50,000	100
B 226	168,000	166,000	8,900	10

* At farm B 93 there was a delay of 2 hours before cooling. Cooling was not very efficient.

The milk from each farm was examined on one day. Later, on the same day, all milking utensils from farms B 12, 31, 93 and 139 were transported to the country depot where they were sterilised by flowing steam for 20 min. and returned to the farm in time for the afternoon milking. The utensils on farms B 523, 527 and 530 were steamed at 210–212° F. for 20 min. in a tin-lined box fixed over an ordinary farm copper. The utensils from farm B 226 were dipped in boiling water, the rubber tubes and teat cups being placed in a cloth bag and immersed for a period of 5 min.

On the day following utensil sterilisation, the milk from each farm was again examined before and after laboratory pasteurisation.

In the cases of farms B 12, 31, 93, 139 and 226 no alteration other than utensil sterilisation was made in the ordinary farm routine methods of milk production. On farms B 523, 527 and 530 a definite effort was made to exclude contamination other than that carried by the utensils.

Return of heat-resisting organisms after utensil sterilisation.

After the original utensil sterilisation of farms B 31, 93, 139 and 226 a return was made to the usual farm routine methods of cleaning. On farms B 31 and 226 the heat-resisting organisms reappeared in significant numbers within 12 days, while on farms B 93 and 139 the milk remained free from heat-resisting organisms for a period of 30 days, after which no further tests were made. The freedom of the milk of B 93 and 139 from heat-resisting organisms after the original utensil sterilisation may be due to greater care

on the part of the farm workers who were aware of the tests which were being made. It is interesting to record that the heat-resisting organisms from these farms were micrococci, having very little effect on milk when grown in pure culture, whereas the milk from farms B 31 and 226 contained large numbers of very active resistant streptococci, capable of clotting litmus milk in 24 hours at 37° C.

DISCUSSION.

Very early in this investigation it was found that by the use of standard agar as a plant control medium, milk was being accepted for processing which was really very unsatisfactory. At the same time efficient bacteriological control of plant processes was not possible without the introduction of a medium more suited to the growth of the bacteria associated with milk. The most suitable medium was found to be standard agar with the addition of 0·5 per cent. sterile milk. This amount was sufficient to support the growth of the majority of organisms common to raw and pasteurised milk, and, at the same time, it had no clouding effect upon the medium.

The excessive numbers of heat-resisting streptococci found in the bulked supplies of raw milk from certain areas have, in two cases, been found to be associated with twice-daily collection of milk from the farm. This system of twice-daily collection was, in one case (depot G), accompanied by the non-cooling of milk at the farm. It has been shown, by experiments, that if milk, containing heat-resisting organisms, remains uncooled for more than 2 hours, rapid multiplication of heat-resisting organisms may take place. In view of this, adequate cooling of milk on the farm should be insisted upon. There can be no doubt that where a supply consists largely of uncooled milk produced by means of unsterilised (although thoroughly washed) milking machines, the pasteurised product will contain excessive numbers of heat-resisting streptococci. These organisms will not be detected if standard agar is used as a control medium, but they will depreciate the hygienic quality and keeping properties of the pasteurised milk.

That the omission of prompt cooling leads to serious trouble and renders the milk quite unfit for use in a pasteurising plant is shown in Tables X and XI. When milk is cooled at the farm to 60° F. the increase reported does not take place.

Another important point of interest has been revealed by the investigation. At the country depot where this work was carried out a bonus system, based on the bacterial count of raw milk supplies, has been in force for a number of years. There are, approximately, 400 farmers supplying milk to the depot. The supplies from 118 farms have been examined and of the 20 which were found to contain more than 30,000 heat-resisting organisms per ml. only three have succeeded in gaining higher than the 200th place in the classified list for the twelve months, October, 1931 to September, 1932. The milk produced with greater care yielded a more satisfactory pasteurised pro-

duct than the milk from farms where there had been some neglect of certain principles of clean milk production. Although no constant relationship has been found between the bacterial counts of the raw and pasteurised milk, there is no doubt that clean methods of milk production exert a great influence on the suitability of the milk for pasteurisation.

Orr (13), Prucha *et al.* (14), Williams (15) and many other workers have shown that, of the various factors that influence the production of clean milk, the sterilisation of utensils is of foremost importance. Farms B 12, 31, 139 and 226 were selected because of the unsatisfactory quality of their milk. By the simple process of utensil sterilisation, accompanied by no other change in the ordinary routine of production, an infection of hundreds of thousands of organisms in the raw milk was reduced to one of less than 20,000, while the number of heat-resisting organisms was reduced from over 100,000 organisms per ml. to less than 100 per ml. These results were obtained during the hottest month of the year and the evening's milk was 18 hours old when tested.

When milk produced by hand is compared with machine-produced milk on the basis of suitability for pasteurisation, there is evidence to show the superiority of the hand-produced milk under ordinary farm conditions. This superiority is probably due to the fact that hand-milking utensils can be subjected to a more thorough cleansing, although it has been shown that, when the components of a milking machine are treated with boiling water or steam between milkings, the milk produced is of exceptionally high grade.

Many instances have been observed where milk of a known high standard of hygienic quality has been found to contain large numbers of bacteria due to the lack of refrigeration, while milk produced under less satisfactory sanitary conditions frequently appears to be of a higher standard when judged by the bacterial count of the raw supply. Bacterial plate counts, as a means of grading and comparing milks, are quite misleading unless all the conditions of age and of time and temperature at which they have been maintained are similar. By means of a bacteriological examination of milk, after laboratory pasteurisation, contamination of the milk by unsterilised or improperly washed utensils is frequently revealed. The value of such a test has been clearly demonstrated in the present investigation.

Experiments on the substitution of a sugar fermentation test for the rather laborious plate-count test are in progress. The inoculation of sucrose solutions with a 1/1000 dilution of laboratory pasteurised milk seems to offer an excellent substitute for the present methods for the grading of milk. This investigation will form the subject of a further report.

Conclusions.

1. Standard agar enriched with 0·5 per cent. sterile milk is a satisfactory plating medium for the control of pasteurised milk.

2. There is a great variation in the number of heat-resisting organisms present in raw milk from different sources.

3. Under ordinary farm conditions and without utensil sterilisation, more heat-resisting organisms are present in machine-produced milk than in hand-produced milk.

4. There is no constant relationship between the number of organisms present in raw milk and the same milk after pasteurisation.

5. Non-cooling of milk at the farm frequently encourages the development of heat-resisting organisms.

6. Heat-resisting organisms frequently originate from the surfaces of unsterilised utensils.

7. Daily sterilisation of milking vessels under the conditions studied has largely succeeded in eliminating the presence of heat-resisting organisms from milk.

8. This investigation shows clearly that a simple quantitative plate count of raw milk affords little information as to the suitability of the milk for pasteurisation.

Our thanks are due to Prof. G. S. Wilson for helpful criticism and to the Directors of United Dairies, Ltd., for permission to publish this work.

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60. DISCOLORATION IN NEW ZEALAND CHEDDAR CHEESE. MUDDY, PINK AND BLEACHED DEFECTS

I. BACTERIOLOGICAL INVESTIGATIONS

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(With 2 Figures.)

During the past two years, discoloration, owing to its prevalence in Cheddar cheese, has been the subject of much investigation by the New Zealand Dairy Division, and in the present paper are incorporated details of the observations made in this laboratory and in cheese stores in New Zealand and England, together with short reports on the same subject from English laboratories.

To avoid confusion it must be emphasised that the defects dealt with are "bleached," "pink" and "muddy" discolorations only. The observations do not apply to "black spot"(1) (due to lead or other metallic contamination), nor to "red spots" (due to faulty annatto), nor to "acid cut" bleaching which seems to follow the outline of the original pieces of curd. Muddy or dark discoloration occurs in white and in coloured cheese, but the bleaching and pink discoloration dealt with here are confined to coloured cheese.

The first sample of dark discoloured cheese was received in August, 1929, and the flora of the affected areas determined. It was found to consist almost entirely of lactobacilli with a few acid-producing cocci and numerous yeasts and moulds. Similar examinations carried out at the National Institute for Research in Dairying in England confirmed these results. It was observed that the discoloration was confined to the neighbourhood of cracks and fissures in the cheese, but the importance of this point was not at first appreciated, and metallic contamination and faulty annatto, rennet and salt were all considered to be possible causes of the trouble.

Experimental addition of copper lactate to cheese milk.

An experiment was carried out in September, 1929, in the dairy attached to the laboratory. On several successive days, 0·5 g. quantities of copper dissolved in lactic acid and neutralised were added to 15 gallons of milk in small experimental vats. The cheese from the experimental and control vats were examined at 3 weeks old and again at 4 months, but no change in colour was seen. Iron likewise gave negative results when used in the same quantity.

Negative findings have also resulted from the introduction of aluminium to milk for cheese-making, according to Mr P. O. Veale at Hawera.

During 1930 large numbers of discoloured samples of cheese were received at the laboratory and more detailed bacteriological investigations became possible. Cultures made from both bleached and normal portions gave the following results.

Lactobacilli, together with a few lactic streptococci, were predominant in all cultures, whether from bleached or normal portions. By the use of plate cultures in selective media such as beer wort agar, Sabaraud's agar, and Czapek's agar, moulds and yeasts were found in large numbers in both bleached and normal portions, yeasts giving the highest counts of from 20,000 per g. in the normal portions to 500,000 in the discoloured areas; while moulds gave counts of from 5000 per g. in the normal portions to 100,000 per g. in the discoloured areas.

The following types of bacteria, other than lactobacilli and lactic streptococci, were occasionally found in certain discoloured areas:

(1) Coccus: white colony, Gram-positive, non-lactose fermenting.

(2) Rod: Gram-positive, non-lactose fermenting.

(3) Rod: Gram-negative, non-lactose fermenting.

(4) Rod: colony hard and tenacious, marked alkalinity but no immediate digestion in milk, Gram-negative, possibly an actinomycete.

(5) Short rod, forming pink colonies on agar, slow liquefier, lactose not fermented, Gram-negative.

(6) *Bact. aerogenes.*

(7) Mixed colonies of rods and cocci occurring in a number of samples, difficult to separate and possibly working symbiotically, both Gram-positive, giving slight acidity followed by digestion.

(8) Yeasts: two types A and B, both non-lactose fermenters.

These organisms merited special attention, for, apart from moulds, which appeared in considerable numbers in all specimens examined, they appeared to be the only variants of the normal cheese flora of occasional lactic streptococci and lactobacilli.

At this stage the oxygen-reducing capacity in cultures and the capacity to reduce annatto was investigated for all the organisms mentioned above, because the bleaching appeared to be due to the reduction of the annatto colour, together with the removal of oxygen from parts of the cheese affected by bleached discoloration. The possibility that an increased reduction of oxygen in certain areas might cause the annatto to lose its colour, and so give rise to the bleached defect, was suggested by various samples received at the laboratory for examination.

It was known that in some of the samples received, the bleaching had developed only a short time before the sample had been submitted for examination. In these cases the bleaching was often not apparent when the sample was unpacked, but when the sample was re-cut and fresh surfaces

exposed the bleached areas were well defined. These, however, also faded out after exposure for 3 or 4 hours.

In the samples from older cheese, in which the bleaching had become a long established fault, fading took place very slowly and in most cases never became entirely restored to the true colour of the cheese.

Preliminary experimental work on the reduction of annatto colour by micro-organisms.

Chemical tests. The liability of annatto to be reduced in colour after addition to dairy produce was tried experimentally by the addition of reducing agents in small quantities of milk coloured with annatto. In all cases a well-marked reduction in colour was noted.

Bacteriological experiments. Standard loopfuls of the cultures already listed were:

A. Introduced into 10 ml. quantities of annatto-coloured sterilised milk, the tubes sealed and incubated at 37 and 30° C. for a period of 2 months.

B. Introduced into the interior of pasteurised processed cheese placed in 6-in. Petri dishes and paraffin waxed to prevent the spread of mould contaminants.

C. Introduced through various portions of the rind in saline suspensions into the body of a sound coloured loaf cheddar by means of a sterile syringe, the site of each inoculation being marked, numbered, and sealed with a hot knife. In addition a culture of *Cladosporium herbarum*, isolated from a more recently received sample of bleached cheese, was introduced in this experiment.

Observations after a holding period of three months.

Culture numbers as listed	Experiments		
	A	B	C
(1)	Colour change, no alteration	Colour change, no alteration	Colour change, no alteration
(2)	Slight reduction	No alteration	No alteration
(3)	Marked reduction	Slight reduction	No change
(4)	Slight reduction	No change	No change
(5)	No change	No change	No change
(6)	Fairly marked reduction	Slight reduction	No change
(7)	Marked reduction	No change	No change
(8) A	Reduction very slight	Marked reduction	No change
B	Reduction very slight	Marked reduction	No change
(9) Mould, *C. herbarum*	No inoculation	Marked reduction	Marked reduction
Control, *Str. lactis*	Very marked reduction	No change	No change

It will be seen from this table that annatto can be reduced by bacterial action under favourable conditions, but that the comparatively dry and close body of a well-made Cheddar does not offer the same favourable conditions that are present in the moister processed cheese and in milk. The mould, *C. herbarum*, however, gave very marked reduction round the site of inoculation in both types of cheese. This was the first indication that moulds might be the cause of the trouble.

DATA ACQUIRED FROM OBSERVATION OF CHEESE SHOWING BLEACHING, PINK AND MUDDY DISCOLORATION.

Discoloration around the trier holes and cracks. Although at the beginning of the investigation of the colour defects mentioned above no connection between them was recognised, it became apparent when large numbers of samples were obtained that certain features were common to all three types. Firstly, the colour fault or discoloration was only to be found around the "open" holes or cracks in the body of the cheese and was never found when the body of the cheese was close and compact. Secondly, the discoloration of the body of the cheese never became complete, but was usually confined to an area from $\frac{1}{4}$ to $\frac{1}{2}$ in. round the circumference of a hole or the border of a crack. Thirdly, where samples were received in large blocks that included the site of an old cheese trier hole, the borders of the trier hole invariably showed (1) marked discoloration, (2) the penetration of mould from the rind between the sides of the trier hole and the returned plug, and (3) the spread of the discoloration, unaccompanied by mould growth, to any hole in the body of the cheese connected with the affected trier hole by a crack, however slight. On the other hand the absence of any discoloration from similar holes unconnected with the trier hole by cracks, though possibly nearer to it than those affected, was also noted.

Penetration of mould from cracks in rind. Shortly after making these observations about dark discoloration, reports were received from London stating that cheese from a certain factory were showing very marked pink discoloration. A strong smell suggestive of hydrogen sulphide was typical of the affected cheese, and at Shinfield Dr Davies(3) demonstrated the presence of volatile sulphides in the cheese. On visiting the factory a cheese was cut which had been made on the same date as those reported from London. The discoloration (bleaching) which was found had not originated from trier holes, as the cheese had never been tried, but an exactly similar process had originated from cracks in the rind. These cracks not only affected the outer rind but penetrated as very thin fractures right into the body of the cheese and linked up with "open" holes. In all cases where discoloration was evident, mould had penetrated the rind by way of the crack for a comparatively short distance, but had caused bleaching to the depth of about $2\frac{1}{2}$ in. into the interior of the cheese, following the outline of the fissure behind each mould-infected crack. However, certain broad cracks on the rind produced no area of discoloration even though they contained a considerable growth of mould. Inspection showed that these cracks, though pronounced on the rind, did not extend to the cheese behind them, and in most cases they did not penetrate beyond the dark brown band adjoining the rind of a coloured cheese. This was particularly noticeable at the corners where pieces of the rind had been damaged or broken away, creating a wound. The cheese on the wound had

dried quickly and formed a secondary rind or scar, which prevented the mould entering to attack the actual flesh of the cheese.

One of these cheese was forwarded to Massey College where it was held and cut 6 weeks later. The fine fissures leading from cracks in the rind had extended considerably and had linked up the many open holes in the cheese, which was extensively bleached (photo, Fig. 1).

Later a cheese from the same factory was received from London showing the pink defect. In this, the older cracks directly connected with the rind showed pink, while the newer cracks showed bleaching, and in some cases intermediate stages appeared. In this particular cheese, the conditions suggested that bleaching preceded pink or red discoloration, and no change in colour ever took place after any area had attained the red state.

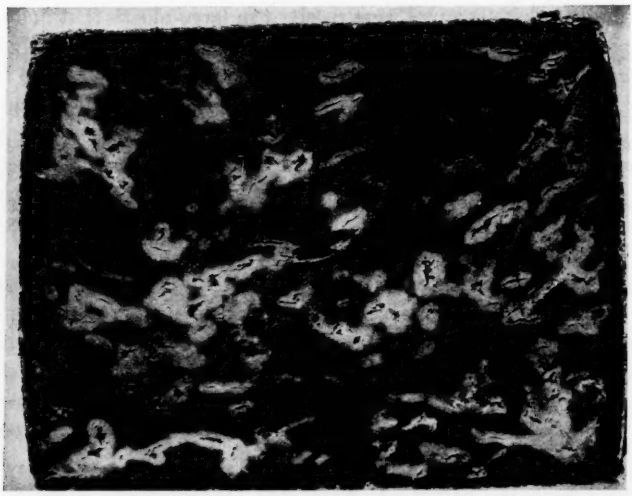

Fig. 1.

Another cheese (made in a different factory) was also returned from London. By the time it arrived the pink had become a bright red, but the cheese showed no signs of bleaching. Mould penetration had evidently occurred through both cracks in the rind and a faulty crown. The latter was probably due to the addition of cold curd to the bottom of the hoop so that a crack had developed right round the circumference of the cheese and a portion had risen like a lid, allowing profuse mould growth to enter the interior. During the next few months, the gradual development of mould in the interior was watched, but the deep red did not change. About the fifth month, a second cycle of discoloration started. A bleached band was seen surrounding all red areas to the extent of about $\frac{1}{8}$ in. Some time afterwards, these bleached portions also became pale pink and finally turned to red.

In addition to these observations made at Wallaceville and at the factories concerned, further interesting information was supplied by Prof. W. Riddet of the Dairy Research Institute, Palmerston North, as a result of a comparison of a series of cheese ripened in vacuum containers with cheese made from the same milk and from the same vat, ripened under ordinary atmospheric conditions. The cheese ripened under ordinary conditions showed considerable dark discoloration, while those ripened *in vacuo* showed a complete absence of discoloration.

Characteristics of cheese affected by bleached, muddy or pink discoloration.

(1) Muddy discoloration may affect both coloured and white cheddar cheese; off flavours and smell only develop after a very long period of ripening, but discoloration often appears in a well-made cheese after 3 months.

(2) Bleaching, a complete local reduction of colour in an annatto-coloured cheese, which occurs rather sooner than dark discoloration, appears to spread more rapidly, and is frequently accompanied by a smell of hydrogen sulphide and bad flavours. When the cheese is stored the bleached areas may change to a pale pink, especially in moist, weak-bodied cheese, lacking in acidity.

(3) Pink discoloration may commence as a very pale flesh pink in annatto-coloured cheese, and progress to a deep red, or it may develop as a second stage to bleaching, usually accompanied by a very bad smell and flavour. When the red stage has been reached no further colour change takes place and the red becomes permanent. Pink discoloration occurs only in annatto-coloured cheese of poor quality and never in white.

The course of development and the location are identical in all three discolorations, and occur as readily in cheese made from pasteurised milk as in cheese made from raw milk.

CHEESE-MAKING EXPERIMENTS.

As a result of the findings already noted in connection with the power of certain bacteria to reduce annatto colour in dairy produce under favourable conditions, certain of the nine cultures previously listed were selected for introduction, by heavy inoculation in saline suspension, into pasteurised milk to be used for experimental cheese-making. The object was to determine whether the cultures could produce bleaching and subsequently pink discoloration in cheese when added in large quantities to the milk; and also to ascertain whether they had any effect in producing muddy or dark discoloration.

These cultures, together with a few common contaminants isolated from cheese cultures and marked with an asterisk, were introduced into pasteurised milk for cheese-making on the dates shown below:

Date	Culture added
July 1	**Bact. subtilis*
July 2	**Bact. aerogenes*
July 3	Mixed colony (rod and coccus apparently in symbiosis)
July 15	Culture No. 2
July 16	*Saline suspension of *Str. lactis* (other than the strain used as starter)
July 17	**Pseudomonas fluorescens*
August 10	Yeast culture No. 9

Each day 30 gallons of milk were pasteurised and half was run into each of two vats. To one vat was added a heavy inoculation of washed cultures of the particular organism, while the other vat was worked as a control. Before each day's making all the equipment used was thoroughly sterilised.

Raw milk of the best quality was selected, the count varying from about 1500 to 4000 organisms per ml. The count after pasteurisation varied from 450 to 1900, while the whey from the control vats never contained more than 900 organisms per ml.

A number of cheese had previously been made in which the same annatto, salt and rennet had been used, without showing any sign of colour blemish. In addition to this, the annatto was tested for lead.

Two cheese were made from each vat, giving a total of twenty-eight. These were graded at the age of 5 months, when each cheese was tried by the same grader with the same trier, but no sign of colour blemish was to be seen.

The series were kept for another 6 weeks and then re-examined. *One* cheese only was found to show marked muddy discoloration on the plug withdrawn. The cheese proved to be an uninoculated control from the experiment carried out with yeast No. 9. The untried sister cheese from the same vat was then re-examined, but showed no trace of discoloration.

Further examination of these two cheese in the laboratory revealed in the discoloured one an old trier hole apart from the two new holes created by recent grading, while only the two new trier holes appeared in the sound coloured sister cheese. Both cheese were then cut in half through the trier holes, and the following interesting features noted. In the discoloured cheese, mould apparently originating from the rind had grown down the old trier hole between the cheese and the sides of the returned plug. Behind this growth of mould lay a thin, very dark discoloured band. Where the mould growth ceased, this dark discoloured band became much lighter in colour but considerably broader in area (to the extent of ½ in. from the edge of the trier hole) and followed the outline of the hole exactly. Where any thin crack, in many cases only just visible, led away from the trier hole into the body of the cheese the discoloration followed this crack to the same breadth at each side of it, and as the crack spread to the "open" holes in the body of the cheese the discoloration followed exactly the contour of the hole, and so spread throughout. The newer trier holes showed no mould growth and no discoloration. At the same time cracks and open holes not connected with the trier hole showed no discoloration even though they were in some cases less than ½ in. away. The cut face of the sister cheese showed no sign of discoloration and no mould penetration either by way of a crack in the rind or from the trier holes.

These facts, together with the conditions under which the cheese were made, suggested that the trouble was not due to the raw materials but to some factors which operated after the cheese had left the press. Only such factors could affect one cheese and not another from the same vat. The evidence showed that the trouble was caused by the breaking of the rind and

the introduction of air at the trier hole whereby mould was enabled to penetrate the body of the cheese. The idea was confirmed by bacteriological investigation, for the flora of the discoloured cheese was the same as that of the sister cheese, and consisted largely of lactobacilli, a few yeasts and penicillia. The mould content of the discoloured portions was, however, very much higher than that of the normal coloured portions.

These facts suggested that after the visible mould growth had ceased, owing to unfavourable conditions, the mycelia of the mould adopted an intracaseous growth, following the cracks and reappearing as a visible growth in various holes in the interior of the cheese where conditions were more favourable. Cultures were repeatedly made from both normal and discoloured areas and showed the presence of a heavy mould count, practically entirely composed of penicillia, in all discoloured areas. This could have arisen from living mould spores that had been unable to germinate, or from portions of mould mycelial strands that would be broken up in making the dilution. Sections of the same material were cut by means of a microtome, and several forms of differential stains were used. The cut and stained sections showed, in some cases, the presence of fragments of mycelial strands at some distance from the seat of the visible mould growth, but, as a whole, this method of examination proved unsatisfactory.

At this stage of the investigation, through the kindness of Prof. Riddet, a number of experimental cheese were made at the Experimental Dairy of the Dairy Research Institute, Palmerston North, with the object of obtaining data on the following points:

(1) The influence of the addition to milk of mould spores of a type of *Penicillium* frequently isolated from discoloured cheese in New Zealand (*P. puberulum*).

(2) The influence of infection by *P. puberulum* spores in cheese plugged at different ages.

(3) The influence of the infection of artificially cracked rinds by the same strain of *Penicillium*.

(1) *The influence of mould spores added to milk.* In the first day's make two vats of milk were used, vat No. 1 for inoculation purposes; vat No. 2 was worked as a control. Mould spores of *P. puberulum* were added in large quantities to vat No. 1 just before rennetting, and care was taken that no contamination should take place in the working of control vat No. 2.

Two weeks after manufacture, one cheese from both control and inoculated vats was plugged, the other cheese from each vat remaining whole.

When the cheese from both vats were cut in half it was seen that discoloration had occurred in all plugged cheese whether from the control or inoculated vats, and had developed from mould growth entering the trier holes from the rind. This led to the conclusion that the infection of milk by mould spores, however heavy the infection, was not of primary importance in the development of discoloration.

(2) *Plugging at different ages.* The second, third and fourth day's make was confined to the manufacture of cheese as free from mould contamination as possible, for the purposes of being plugged at different stages of maturity, *i.e.* at the age of 1, 2 and 3 months.

At the time of plugging, each plug as it was removed was dusted heavily with spores of *P. puberulum* and then carefully replaced in the cheese, and one cheese from each batch was allowed to remain as control.

The cheese were all cut in half when 5 months old, in such a way that the cut crossed each trier hole.

On examination it was found that all plugged cheese were discoloured (except those in which the plugs had been so carefully replaced that no mould could enter from the rind) at whatever stage of maturity the plugging had taken place; the control cheese showed no trace of discoloration. It was also found that mould infection had not occurred from the mould spores dusted on each plug but had apparently entered the trier hole from the rind. Visible mould growth was apparent not more than $\frac{1}{2}$ in. from the rind, after which typical discoloration commenced. No trace could be seen of the mould spores artificially introduced.

This led to the conclusion that mould spores introduced by means of plugging did not play an important part as a cause of mould infection in the interior of the cheese, but that the trouble was mainly caused by the entrance of moulds, already established on the rind, by means of cracks or trier holes. It was found on examination that the moulds established on the rinds of these experimental cheese were similar to those artificially introduced.

(3) *Mould infection of artificially created cracks.* No data could be obtained on this point, as difficulty was found in creating the type of cracking found to lead to discoloration in export cheese. Artificially created cracks dried out too quickly and formed a hard rind behind the fracture, so that no fine fissure for the introduction of mould and air extended into the body of the cheese.

In addition to these negative results positive evidence was obtained showing the part played by the entry of mould from the rind through crevices of the trier holes. The appearance of the mould growth in trier holes emphasised the fact that such development was due to mould mycelia creeping in from the rind rather than to the germination of the spores with which the trier holes had been inoculated.

In every case where the plug had been replaced in the trier hole so as to seal it effectively and prevent the entry of mould there was no discoloration associated with the trier hole. On the other hand, whenever the mould had been able to creep into the trier hole and grow within, bleaching occurred in the vicinity, whether the cheese was from an experimental or from a control vat.

USE OF METHYLENE BLUE AS AN OXYGEN INDICATOR.

Another experiment consisted of adding methylene blue instead of annatto to a small vat of milk. One of the two resulting cheese was tried soon after the removal from the press, while the other was tried about a month later. After 4 months, these cheese were cut in half through the trier holes. Most of the cut surface was uncoloured but the blue areas gave a perfect map of the oxygen circulation within the cheese. Around the rind was a blue margin about $\frac{1}{4}$ in. wide. In addition. the blue extended the sides of the trier hole, along all cracks leading away from it and around holes connected with these cracks. There was, however, little blue adjacent to the seat of the mould growth

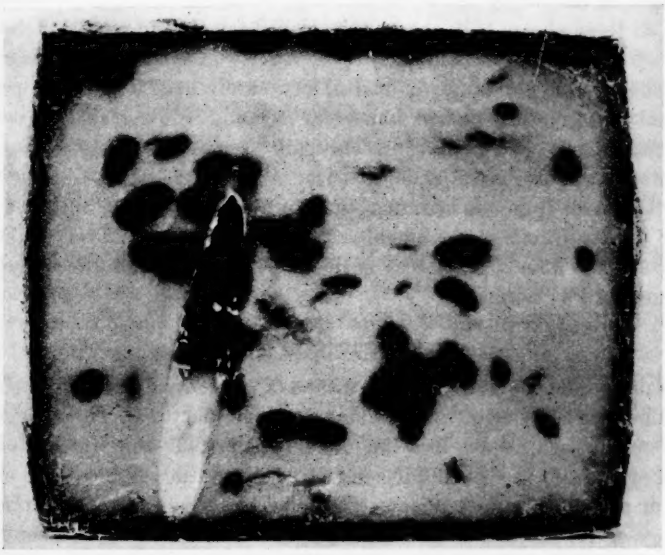

Fig. 2.

in the trier hole. The blue areas were actually quite similar in distribution to the bleaching which had frequently been observed after the growth in trier holes (photo, Fig. 2).

The fact that oxygen is essential for the extension of the mould mycelia within a cheese is also confirmed by information reported to the Department by Prof. Riddet in connection with an experiment upon the storing of cheese in tins. At a certain factory on several days three cheese from the same vat were stored, one in the ordinary crate, one in a sealed tin and one in a tin which was evacuated prior to sealing. When the cheese were examined at the age of 8 months it was found that, as a result of mould growth, dark discoloration appeared in some of the cheese held in crates, while there was no discoloration in the corresponding cheese in tins with or without vacuum.

With the object of discovering whether cheese might be predisposed to discoloration by some peculiarity of any individual supplier's milk the experimental factory equipment was sent in September, 1931, to a factory which had experienced serious trouble with discoloration in the previous season. One vat daily was made in turn from every supplier's milk and of the two resulting cheese one was tried, while the other remained untried. When these cheese were cut after 4 months, out of a total of 47, 20 which had no old trier holes were not discoloured in any way; 2 with old trier holes, in which the plug had been replaced so as to prevent the entrance of air and mould, were not discoloured; while 18 were more or less discoloured and in every case showed mould growth which had crept down the trier hole from the rind and developed in the cavity at the bottom of the hole.

Of all these cheese only one had developed faintly the pink and the associated off flavour which had troubled the factory in the previous season. The sister cheese from the same vat had unfortunately been disposed of prior to the final cutting of the cheese, but as far as could be ascertained showed no defect in colour, smell or flavour. The pink cheese had a very strong smell suggestive of hydrogen sulphide, and lead acetate test paper became brown where the gases from cracks impinged upon it. These defects appeared to be due to the conditions of the mould growth in the cheese and not to the particular supplier's milk, for cheese made from the same milk a few days later was in every way normal.

Preventive measures.

To determine the most suitable means of preventing the development of mould in trier holes, eight loaf Cheddar were manufactured. Three plugs were taken out of each cheese, in a line; each plug or plug-hole was dusted with mould spores and the whole plug immersed in solutions respectively of salt, boric acid, and sodium carbonate. The plugs were then carefully replaced in the cheese. At the end of 4 months all cheese were cut through the row of trier holes, but it appeared that none of the spores which had been originally dusted on the plug had germinated, as mould growth did not extend for more than $\frac{3}{4}$ in. from the rind of the cheese whereas the plugs had been dusted for their entire length. Nevertheless in no case had the preserving solution prevented the entrance from the rind of mould, which crept down the trier hole to the depth of about $\frac{3}{4}$ in. This mould growth failed to produce any spread of discoloration because the cheese were extremely close and rather dry. However, at the corner of one of the cut cheese was a marked area of bleaching, which, by dissection, was discovered to originate from an old mould-infected crack leading from the corner of the rind.

Experiments were carried out on the prevention of mould development in trier holes by two further methods. The first, developed by Udy, was to use a large grease gun to introduce minced cheese into each trier hole. This method effectively filled the trier holes and prevented mould growths, but

such a method could not be suitable for use in the grade stores. In the second method, proposed by Massey College, a high-grade paraffin wax was used to fill the trier holes. This method successfully prevented mould growth in most cases but was considered to be unsightly and not likely to be acceptable to the trade. Moreover, in one case a small cavity remained unfilled at the bottom of the trier hole so that a small development of visible mould growth occurred, actually no larger than a lead shot. This was responsible for a very large area of discoloration in adjacent portions.

It was finally found that the trier holes could be most easily sealed by brushing on a little melted paraffin wax. Sometimes this may crack off, but the convenience and success of the method has led to its adoption in all New Zealand grading stores. In addition the excessive boring of cheese at any time is to be avoided.

During the present season a factory (one of the largest in the Dominion) which experienced serious trouble and loss due to discoloration last season, received special supervision and advice based on the foregoing observations. Before the manufacturing season commenced the factory and curing room were thoroughly disinfected. Special care was taken to see that the cheese were well scalded in the press so that intact rinds were produced. The production of cracked rinds due to careless handling was also minimised. Present reports indicate that this factory has practically eliminated the trouble of discoloration due to cracked rinds.

During recent seasons very many complaints have been made in Britain about the damage done to cheese through mould penetration from trier holes and cracked rinds, and the losses involved must have amounted to hundreds of pounds annually. It appears that if the suggestion arising from the above investigations can be applied in factories and grading stores throughout the Dominion these complaints and accompanying serious losses can be eliminated.

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61. DISCOLORATION IN NEW ZEALAND CHEDDAR CHEESE. MUDDY, BLEACHED AND PINK DEFECTS

II. BIOCHEMICAL INVESTIGATIONS

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When the writer began to investigate this subject much bacteriological work had already been carried out. This work, which has been discussed in the previous paper, eliminated a large number of possibilities and showed that the chief biological abnormality was the count of moulds and yeasts, which were usually much more numerous in discoloured portions than in normal portions of the same cheese. No evidence was available to show whether or not the biochemical reactions of the discoloured cheese agreed with the mould theory which was first put forward by Morgan after much careful work.

Since mould-ripened cheese, such as Stilton, is well known to develop a rather high *p*H, the study of this property seemed to be important. The *p*H of normal and of discoloured portions of the same cheese has been compared in a large number of cases, using the quinhydrone method (1). The results fall into three classes: first, the *p*H of pink portions is usually close to that of normal. The significance of this will be discussed later. Second, the bleached portions have often a distinctly more alkaline *p*H than the normal, though occasionally the differences are slight. As a rule, slight differences are obtained when the adjacent mould growth (in trier hole or cracked rind) has not been long established, but when the mould has obviously been growing for a considerable time greater differences are noticeable. These well-developed growths are usually distinguished by a muddy discoloration close to the region from which the mould appears to have spread. In such cases, a third type of result has been obtained—the muddy portions close to the seat of mould growth are always much more alkaline than even the most alkaline bleached portions. In his study of cheese moulds Neill prepared a series of sterile slices of cheese in Petri dishes. These were inoculated with various strains of the two principal mould types found in New Zealand Cheddar cheese. After a month's growth *p*H determinations were made by the writer. The *p*H had risen from 4·8 to values in the vicinity of 6·7 with slight but immaterial differences between the strains.

As cheese matures the original lactic flora (and most of the contaminating types) die off and are replaced by the lactobacilli. Neither the latter nor the former possess catalase, *i.e.* they do not decompose hydrogen peroxide and liberate oxygen; but when hydrogen peroxide is applied to discoloured cheese

much frothing at once occurs. In order to obtain a more quantitative measure of this the writer fitted up a simple apparatus consisting of a 20 ml. test-tube with a rubber cork through which passed a tube about 20 cm. long. The tube was drawn out to a small opening at its lower end, and was of such a bore that when graduated in ml. the marks (made with a diamond or file) were about three linear centimetres apart. One-gram portions of normal and of discoloured cheese were dissected out and placed in such tubes, together with 10 ml. of warm sterile water (30° C.) which enabled the cheese to be easily macerated with a sterile glass rod. Having thus prepared a set of tubes, 1 ml. of 3 per cent. hydrogen peroxide was added to each, the stoppers were quickly inserted, and the inner tubes adjusted with the liquid at a suitable mark. The tubes were kept at a uniform temperature and were examined 2 hours later. In those with a high catalase content the evolution of oxygen forced the liquid up the inner tube, and the extent of this rise was an approximate measure of the catalase content. Normal cheese usually gave only a slight reaction, while discoloured portions close to the seat of mould growth always had a high catalase content. In general, the more the *p*H of the discoloured cheese differed from the normal the greater the catalase reaction, as shown in the accompanying table of a few typical samples.

Table I.

Cheese No. and condition	Normal *p*H	Discoloured *p*H	Discoloured Colour	Discoloured Catalase reaction
1. Old and dry	5·34	5·34	Pink	—
	5·22	5·15	Pink	—
2. Mature	5·30	5·44	Bleached	—
		5·45	Pink	—
3. Good condition	4·91	5·23	Bleached	—
		6·36	Muddy	—
		6·45	Muddy	—
4. Extensive bleaching	5·06	5·17	Bleached	Slight
		5·21	Bleached	—
		6·09	Mouldy	—
5. Six months old	4·82	5·11	Bleached	Strong
Six months old (near trier hole)		5·35	Mouldy	—
6. Six months old; bad flavour	4·99	5·05	Pink	—
	5·01	5·04	Pink	—
		5·01	Pink	—
		4·94	Pink	Slight
		4·87	Pink	—
7. Mature	5·13	5·44	Pink	Strong
Mature (near mould)		7·16	Muddy	—
8. Mature	5·30	5·29	Pink	Very slight
		5·40	Bleached	Very slight
		6·92	Muddy	—
	5·37	5·37	Pink (adjacent)	—
9. Five months old; slight off flavour	4·97	4·87	Bleached (? faint pink)	—
	4·97	4·91	Bleached (? faint pink)	—
10. Mature; high fat, 40 %	4·98	4·98	Bleached	—
	4·96	4·87	Bleached	—
11. Mature	5·25	5·20	Bleached	—
	5·25	5·22	—	—

Yeasts, as well as moulds, contain catalase and have frequently been obtained from discoloured cheese. Whether they modify the mould growth or not, they do not appear to be able alone to produce the discoloration. The evidence of *p*H determinations and catalase tests thus seems to support the theory that moulds are very closely associated with the various types of discoloration.

In further considering the chemical aspect, first the bleached defect and then the pink will be discussed, since both involve changes in the normal annatto colour of the cheese.

Bleached discoloration.

Comparatively little is known of the chemistry of annatto. Thorpe (2) states that bixin, the essential principle, is red and very sparingly soluble, but alkalis readily dissolve it, giving the well-known orange-red colour. It can be reduced by the action of zinc dust and acetic acid to an orange-coloured derivative which, on standing in air for a few days, becomes oxidised to a colourless substance. Test-tube experiments with the commercial colour show that it is easily bleached by both oxidising and reducing agents. The conditions within a cheese are, however, highly reducing in nature and, even though oxygen may be present in certain parts (as shown in the methylene-blue cheese), these reducing conditions predominate. That this is the case even in some bleached portions is shown by the catalase reaction observed. According to Hewitt (3) the addition of catalase to a culture containing peroxide causes an immediate and rapid fall in potential despite the resulting vigorous oxygenation. A high catalase content would thus appear paradoxically to be able to liberate molecular oxygen and at the same time maintain a high reducing intensity.

In a few cases (Table I, Nos. 9, 10, 11) the *p*H of bleached portions has been observed to be a shade lower than that of the adjacent normal. One cheese in which this occurred had a particularly high fat content and the cracks in it were lined with pockets of fat. Thus arises the possibility that the bleaching along the margin of the cracks is related to oxidative changes in the fat. Davis states: "At the surface of the cheese and along borings and cracks in the cheese, zones of higher oxidation potential exist (4)." The methylene-blue cheese experiment recorded by Morgan showed that the cracks connected with the mouldy trier hole contained oxygen, *i.e.* were at a high oxidation potential (*r*H above 12). Hence the cheese colour may be thought to be bleached by oxidation, but on the other hand we have frequently observed that on exposure to air the intensity of bleaching fades rapidly. Under these circumstances, the oxygen of the air apparently restores the colour removed by what would appear to be reduction. Thus the colouring matter might be considered as the hydrogen acceptor of an oxidation-reduction system involving the fat. Once changes of this nature had been initiated close to the seat of the mould growth a rapid spread along the edges of communicating

cracks might occur. As Davies writes: "Most workers are agreed as to the formation of an oleic-peroxide which is a relatively potent oxidising agent either as a catalyst (oxygen carrier) to further oxidation, or as an intermediate compound in a series of reactions....Moisture aids the reaction....It has been established that, once incipient deterioration sets in, the onset of taint development increases rapidly, showing that the products as they are formed catalyse the oxidation so much that even the diffusion of volatile products from an oxidising fat can initiate oxidation in an adjacent sample of 'sweet' fat(5)."

Pink discoloration.

In experiments upon annatto attention was devoted particularly to reducing agents. In trying to reduce it under mild conditions so that the colour changes could be followed, the writer dipped a piece of filter-paper in dilute annatto, and exposed it while moist to sulphur dioxide fumes. The paper rapidly became pink. Hydrogen sulphide, which is less soluble in water and therefore not so rapidly absorbed by moist filter-paper, also produced the pink, but more slowly. This effect was assumed to be due to reduction, until acid fumes, such as acetic or hydrochloric or even nitric, were also found to be equally effective. The pink substance is evidently the insoluble bixin which, on treatment with strong soda, becomes the yellow-brown sodium bixinate responsible for the annatto colour. Pink in Canadian Cheddar cheese was recently traced by Hood and White(6) to the use of potassium nitrate during manufacture. But nitrates have not been found when tests have been made at the writer's laboratory upon cheese from factories which have recently experienced pink discoloration. Special tests have been made of the materials used by one factory which experienced most trouble, and neither nitrates nor nitrites were found in significant amounts. The defect found in Canadian cheese may perhaps be the result of changes involving the reduction of nitrate to nitrite, followed by the liberation of strongly acid nitrous fumes which would readily turn the annatto pink.

Experiments were carried out to determine the state of the colour in a cheese which was returned from London on account of the bright pink areas in it. Portions of the pink and of the normal annatto-coloured cheese adjacent to it were carefully dissected out and placed in separate dishes to which sufficient distilled water was added to enable the contents to be thoroughly macerated. On filtering the mixture, the normal annatto-coloured cheese gave a pale, but distinct, yellow filtrate, while that from the pink was colourless and the residue on the filter was still a distinct pink. The insoluble pink would therefore appear to be produced in the cheese by the development of a strongly acid reaction.

The suggestion that the pink is due to acidity conflicts with the evidence of *p*H determinations which reveal more or less alkaline conditions associated with mould growth in bleached cheese. To demonstrate acid production,

neutral litmus agar plates were prepared and inoculated with *P. puberulum*. Large Petri dishes were used and were rendered partially anaerobic with cotton-wool, pyrogallic acid and soda and sealed with wax. Acid was quite distinct especially in the early stages of growth and even when the central portions of the colonies became a deep blue a red acid ring persisted at the growing tips of the mycelia.

In the table of *p*H figures for discoloured cheese several values for pink portions are given. Occasionally these are distinctly higher than normal; in No. 7, this is accompanied by a strong catalase reaction suggestive of a mature mould growth. Usually, however, the *p*H of pink portions is the same as that of normal or is only slightly higher, though in some cases the pink has a slightly lower *p*H. This is most noticeable in No. 6 in which the visible mould growth (in a trier hole) was slight and apparently fairly new. Although the figures referred to show distinct local variations, their reliability can scarcely be questioned, as in each case the values were very carefully obtained with two (sometimes three) electrodes which rarely differed by as much as one millivolt. Lead acetate test paper showed that considerable quantities of hydrogen sulphide or volatile organic sulphides were present, and these probably accounted for the bad flavour possessed by the cheese. In this case, the mould was apparently in the same acid-producing early stage of growth which is responsible for turning litmus agar plates red.

If the cheese is turned pink by acid, the normal colour should be restored by treatment with alkali. This cannot be satisfactorily done by immersing the cheese in an alkaline liquid, as part of the cheese dissolves and bleaching occurs. To avoid this difficulty, a piece of cheese with distinctly pink areas was placed in a small, uncovered Petri dish which was set inside a larger covered Petri dish containing a little strong ammonia. The alkaline fumes caused the pink to disappear and in less than half an hour the whole piece regained the normal annatto colour. Since ammonia is slowly produced as cheese matures, traces of ammonium sulphide may be formed. Annattoed paper dipped in *alkaline* ammonium sulphide remains yellow, but becomes pink when dry. The yellow colour is temporarily restored by strong ammonia fumes, but the pink returns on drying with gentle heat. In order to discover why the development of alkaline conditions due to mould growth does not similarly disperse the original pink the writer devised a simple experiment. Filter-papers were dipped in dilute annatto and partially dried. Some of these were treated with hydrogen sulphide gas in a tube until they became a bright pink. Several phosphate buffers were made up, extending from about *p*H 4 to 8, and portions of each were placed in the two sets of tubes. The yellow papers at once became pink at the lower *p*H values but this faded up to *p*H 5·3. The pink was doubtful above this and at *p*H 6·5 was definitely absent. On the other hand, the pink papers maintained their shade quite definitely from *p*H 4 to 5·9, while even at *p*H 6·9, where the yellow could be definitely seen, a trace of pink still remained. These experiments demonstrate two things.

First, once the insoluble pink has been produced a high degree of alkalinity is necessary to convert it back to the yellow colour. Cheese is usually adequately buffered to prevent sufficient alkalinity being rapidly produced by mould growth to remove the pink. Second, the *p*H of normal Cheddar cheese (about 5·0) is quite close to that at which the pink shade develops in annatto.

Evidence supporting this second contention is provided by the loose, extra bandages which in some factories are placed between the cheese cloth and the hoops. The whey which drains out of the cheese in the press develops a very high acidity, with the result that if annatto is present these bandages are soon coloured a distinct pink. In spite of the fact that they are boiled daily before use they retain this apparently insoluble colour. When a piece of one of these bandages was treated with ammonia fumes the pink was replaced by a pale shade of annatto.

The relation of the pink to the bleaching has given rise to some discussion. In one particular case, a cheese was examined which had such badly cracked rinds that, in spite of the waxing, moulds entered easily and caused an extensive bleaching around the cracks throughout the body. After rewaxing and holding at a rather warm temperature for about 3 months, the cheese was again cut, but the areas around the cracks instead of being bleached were beginning to turn pink. Perhaps the mould was then growing under such unfavourable conditions that it began to produce more acid, and so caused the pink.

Although the evidence so far obtained suggests that the pink discoloration is produced by acid, the assertion cannot definitely be made that the acid in question is hydrogen sulphide. This gas may not even be responsible for the bad flavour often found in pink cheese. If mould growth can initiate oxidation of the fat so as to give rise to bleaching, under slightly different conditions the changes in the fat may perhaps set up sufficient acidity to produce the pink. Moreover, the pink defect is usually associated with much more pronounced off flavours than are found in bleached cheese. These flavours may perhaps be due to the development of ketonic rancidity, of which Davies writes: "If the acidity of the product is such as to liberate the lower volatile fatty acids into the water films, some of these acids might be broken down to give the ketones. An association of the two types of moulds or of yeasts, bacteria, and moulds, might be more powerful in bringing about this type of rancidity than would dry moulds alone (5)." In this connection it is significant that over-moist cheese (often showing free moisture in the cracks) appear more prone to discoloration.

Muddy or dark discoloration.

The chemical nature of the dark or muddy pigment has yet to be explained. It has been found that after prolonged growth on carbohydrate media practically free from organic nitrogen, moulds can produce a dark colour, which may be similar to the pigment they produce in cheese. On the

other hand the following fortuitous observations may help to explain the matter. A faint pink was noticed in a mature *white* cheese which, after use for another purpose, had been cut in half and then waxed. On recutting, the pink was found to be in evidence throughout, but was rather more marked towards the rind. The cheese was set aside to be re-examined next day, and, as was expected, those portions which had been exposed to light and air were no longer pink but had developed a muddy brownish shade which was strongest where the pink had been most pronounced. On one face which had lain overnight in contact with the bench, whereby free access of air was prevented, the change of the pink to the brown had progressed much more slowly. At the outer edges the brown had commenced to appear, but the pink remained especially strong in one or two areas adjacent to cracks which were obviously connected with seats of mould growth. This pink is distinct both in shade and in nature from that found in coloured cheese.

The free amino-acid tyrosine is well known to be present in mature cheese, and various workers (7) have shown that under suitable conditions this substance can be changed by the action of the enzyme tyrosinase to a pink or red substance, which in turn is readily oxidised to a dark brown or black pigment known as melanin.

In England investigations (8, 9, 10) have been made upon Stilton cheese affected with brown or dark discoloration. Cheese showing this defect were found to contain a variety of more or less common organisms (including *B. coli*, *B. proteus*, etc.) which produced similar pigment when grown upon media containing tyrosine and like substances present in mature cheese. After preliminary lactic bacterial action Stilton cheese is ripened to maturity principally by mould action. Later researches (private communication) showed that discoloration was more likely to arise in cheese which had been made most alkaline (by mould growth). In Cheddar cheese showing muddy discoloration we have repeatedly observed foci of mould infection where the *p*H is much less acid than the body of the cheese. The development of the lactic acid bacteria is well known to produce an acidity which restricts the activities of other types. The establishment of mould growth, by locally reducing this protective acidity, no doubt permits a variety of other organisms to flourish. Under these circumstances the centres of mould growth can become foci for the production and distribution of a variety of decomposition products and enzymes. The latter especially may produce dark discoloration and other quite unusual changes in the adjacent cheese.

Complete explanation of all the biochemical changes involved may be difficult to obtain, but meantime ample evidence has accumulated to show the need for excluding mould growth from the interior of Cheddar cheese. Close-textured cheese without excessive moisture provide little scope for the spread of mould within. Open cheese can be protected if care is taken to make good rinds and keep them in good condition. The sealing of all trier holes is essential. These and other preventive measures have been consistently recommended

by the Dairy Division in New Zealand for some time. Factories which have been seriously troubled with discoloration in one season have usually adopted these recommendations to such an extent as to minimise the trouble in succeeding seasons.

Summary.

1. The evidence of *p*H and catalase tests confirms the view that mould growth is closely associated with the production of discoloration in cheese.

2. The pink discoloration appears to be the result of acids acting upon the annatto colour.

3. Evidence available from observations and other sources, suggests that the bleaching is chemically a reduction in which changes in the fat play a part.

4. The muddy or dark discoloration appears to be produced by enzymes (including possibly tyrosinase) which diffuse into the cheese from centres of mould growth and act upon chemical substances present in mature cheese.

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62. VARIATION IN THE COMPOSITION OF MILK IN CERTAIN MIDLAND DISTRICTS OF ENGLAND DURING THE YEARS 1923–31

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(With 12 Charts.)

The quality of milk produced in the various dairy-farming districts of England is a matter of considerable importance to all engaged in the milk industry. During the past 50 years or so, thousands of samples of milk have been analysed each year, but in many cases the data have not been published or rendered available to the general public.

Richmond (1) has given the average composition of milk for 330,000 samples analysed in the laboratory of the Aylesbury Dairy Company over a period of 20 years, but this data, it is believed, refers only to supplies collected prior to 1916. There is no evidence as to the districts supplying the milk from which these samples were drawn, but it is assumed that the bulk of the milk was produced in the Home Counties. The mean monthly variations in composition are given but no figures are available showing the variations in quality year by year.

Apart from the Aylesbury Dairy Company's data, no great mass of figures relating to the composition of milk, as received by milk depots in this country, has been published. One must not omit to refer to the data published in the annual reports of local authorities (under the Food and Drugs Act), but the samples to which such figures refer are drawn, in the majority of cases, from churns or cans of milk, and such samples, on the whole, do not represent the bulked milk of many cows or many herds.

Dairy Companies, purchasing large quantities of milk day by day, are regularly sampling the milk as received and bulked, and the results obtained in their laboratories should throw considerable light on the monthly and yearly variations in the quality of milk produced in the areas from which their supplies are drawn.

The data on which this report is based have been collected in some twelve of the Northern Area depots of the United Dairies, Ltd., during the period 1923–31, and, through the courtesy of the Directors, the writers have been permitted to summarise and analyse this data and place the salient points arising at the disposal of all interested in dairy education and research.

Methods of sampling and analysis.

At each depot the supplies of milk, as collected from the farms, are received into bulking or balancing tanks, from which samples are drawn at intervals, such intervals being based upon the rate at which the milk passes into the larger store tanks for manufacture or dispatch. The sampling periods may vary from 5 to 60 min. according to the quantities of milk passing through the tanks.

The fat percentage is determined by the Gerber method and the percentage of solids-not-fat calculated from the gravity and fat percentage figures. Periodically, control samples are taken and analysed by gravimetric methods.

From the results of these tests a bulk average figure is calculated and, subsequently, weekly and calendar monthly averages are compiled.

The above procedure has been uniformally carried out at all depots, under the direct supervision of one of the authors.

Area of supply.

The farms supplying the depots are situated mainly in the counties of Cheshire, Staffordshire, Derbyshire and Leicestershire. Although the number of farms involved varies from year to year, it can be stated that, on an average, 1800 farms and 20,000 cows have contributed to the data on which this paper is based. The number of samples collected during the nine-years' period is approximately 300,000.

The area of land stretching from the Welsh borders across the Midlands is essentially devoted to dairying and has long been noted for its contribution to the milk production of the country. One has only to recall the names of several well-known English cheeses—Cheshire, Derby, Leicester and Stilton—originating in this area, to realise that this district is well suited to milk production.

Whilst many soil types are to be found throughout this area, a rough survey of the particular sections of these counties from which milk supplies have been drawn, indicates that considerably more than half—probably two-thirds—of the land overlies the Keuper Marl formation. The soils on this foundation are rarely deficient in lime compounds, and this point is one of dominant importance in milk production.

Climatic conditions vary somewhat as one passes from west to east, but it appears to be difficult to assess this factor in measurable terms, without going through records of the various stations in great detail. Unfortunately, few official recording stations were to be found in the immediate vicinity of the depots. It was finally decided to utilise the rainfall and temperature data from the Hoylake and Wistantow stations as representing the western side, the Mayfield, Coventry and Rugby stations for the central portions and the Belvoir and Sutton Bonington stations as representing the more easterly

portions of the area. It is hoped that the average figures from each of these groups of stations, respectively, will give some indication of the climatic differences between the various districts covered by this paper.

Analysis of data.

Variation in the composition of milk is due to a variety of factors, but in the analysis of the commercial data of the type dealt with in this paper, it is not possible to examine the influence of many of these factors individually. It was decided, therefore, to tabulate the data, with the object of throwing light upon:

(*a*) Variation in the composition due to district.
(*b*) Variation in the composition due to month of year.
(*c*) Variation in the composition due to year.

The various areas supplying the depots were divided into three sections, and the following is a general description of each section.

Cheshire section (South Cheshire and parts of Flintshire and North Shropshire).

Formerly the dairy farming in this area was almost entirely given over to farm cheese-making, but in recent years this district has been increasingly drawn upon for liquid milk supplies. In cheese-making districts the bulk of the milk is produced in the summer (grass) months, but since the gradual change from cheese-making to milk-selling commenced, there has been a gradual levelling up of production as between summer and winter. This levelling up has been distinctly noticeable during the period under review.

Although this gradual change in management, entailing a wider distribution of calving periods, is still going on, there is yet a much wider divergence between the quantities of summer and winter milk received in this district, than obtains in either of the other two sections.

Staffordshire section (North Staffordshire).

For many years this district has constituted a valuable source of liquid milk and, in consequence, there are comparatively few farmer cheese-makers. As a result, one finds a more uniform production as between summer and winter, and calving periods are distributed more evenly throughout the year.

Derby and Leicester section (parts of South Derbyshire and North-west Leicestershire).

The management on the farms in this section is very similar to that in the Staffordshire district, with perhaps still fewer farmer cheese-makers. Supplies received at the depots in this section fluctuate the least of any of the three sections of the area under review.

Discussion of results.

For brevity and clearness, the results of analysis of the data are presented in graphical form.

Variation in fat content.

(1) *Yearly variation* (Charts I and II).

On examining these graphs one observes that in 1925, 1926, 1928 and 1929 the average fat percentage of the milk from all depots was below the normal

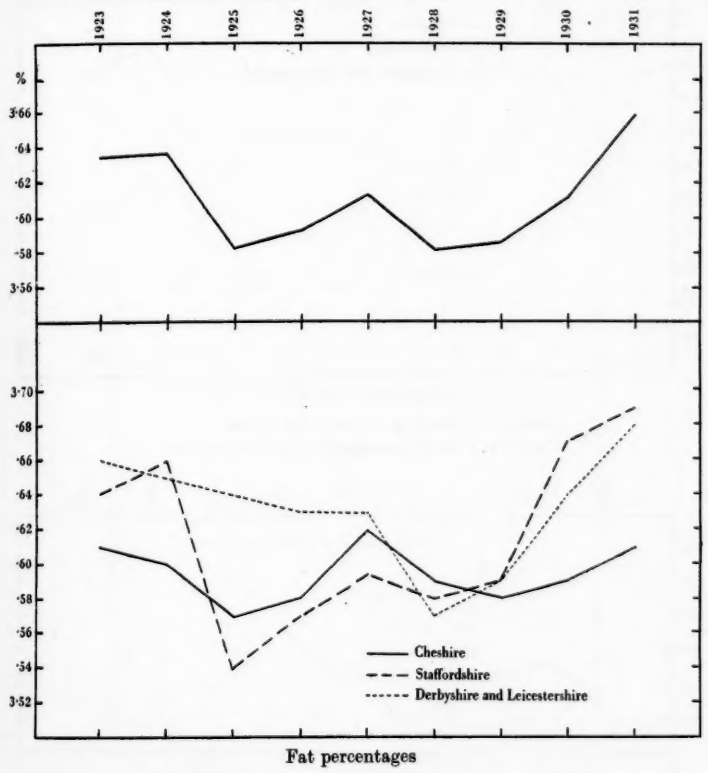
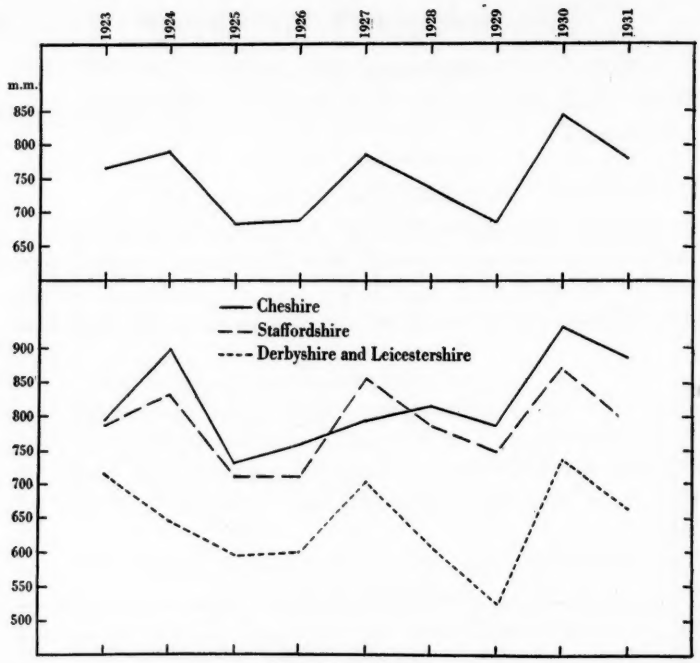


Chart I. Yearly averages for all areas.
Chart II. Yearly averages for individual areas.

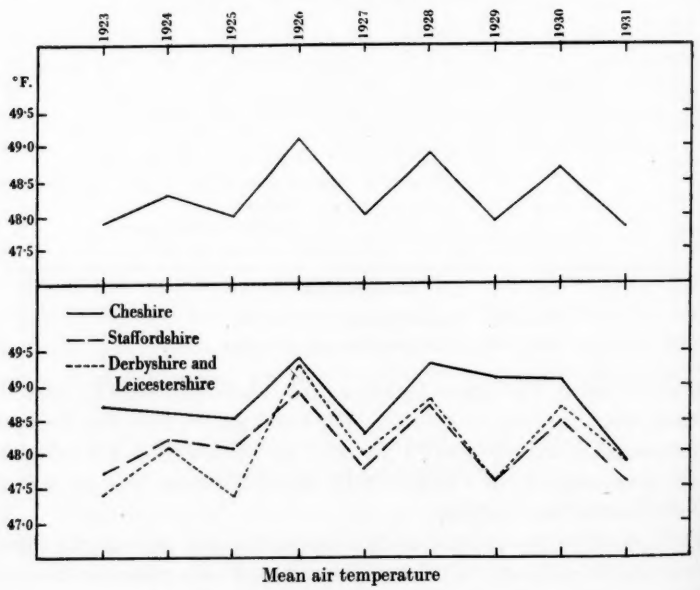
of the whole period. The figures for the separate districts (Chart II) show some departure from the general average. For example, in 1925 the Derbyshire and Leicestershire average was 0·1 per cent. higher than that of Staffordshire. The fat percentage of the Cheshire milk varied less from year to year than those of the other two districts.

The cause of yearly variation of the average fat percentage may be climatic, and it is rather significant that the graph in Chart I follows closely the average rainfall graph (Charts III and IV), which is based on data recorded at stations



Rainfall in millimetres

Chart III. Yearly averages for all areas.
Chart IV. Yearly averages for individual areas.



Mean air temperature

Chart V. Yearly averages for all areas.
Chart VI. Yearly averages for individual areas.

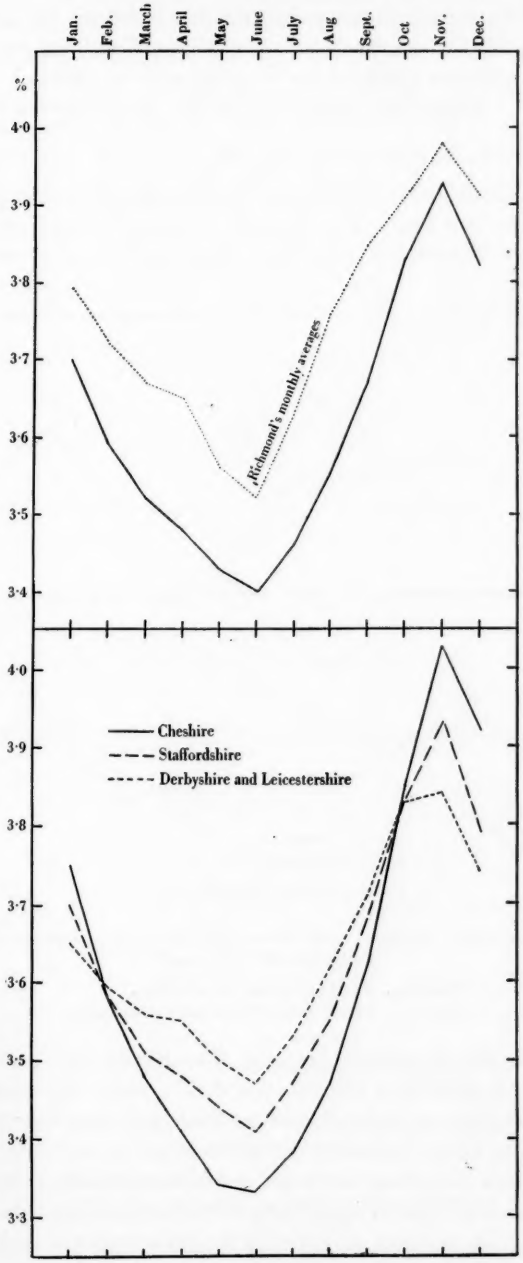


Chart VII. Monthly averages for all areas.
Chart VIII. Monthly averages for individual areas.

within, or in the vicinity of, the various districts supplying the milk. The only year in which the two graphs do not agree is 1931. The mean air temperature figures for the various years are shown graphically in Charts V and VI, but no correlation between these means and the fat figures appears to exist.

(2) *Monthly variation* (Charts VII and VIII).

In Chart VII is shown the average monthly fat percentage for the whole area during the nine years. For comparison, a graph based on Richmond's average monthly figures (2) is given. The extreme months—June and November

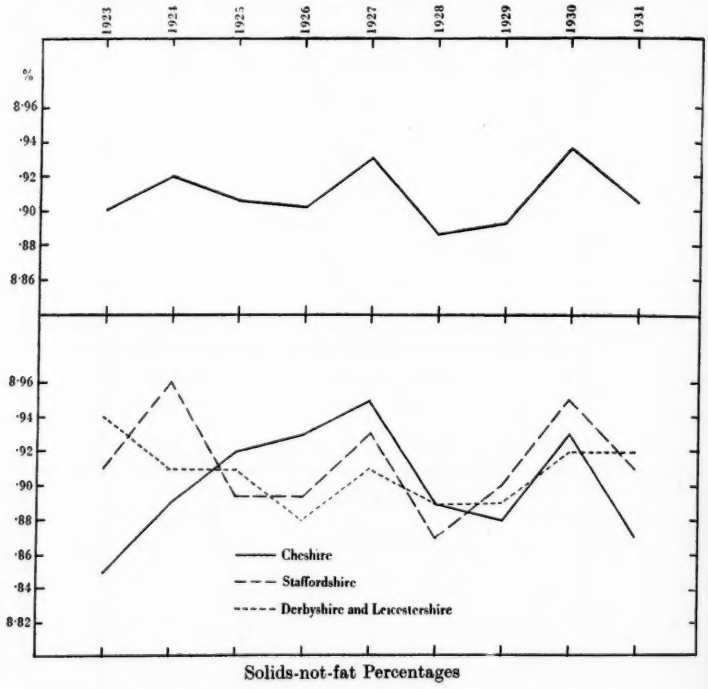


Chart IX. Yearly averages for all areas.
Chart X. Yearly averages for individual areas.

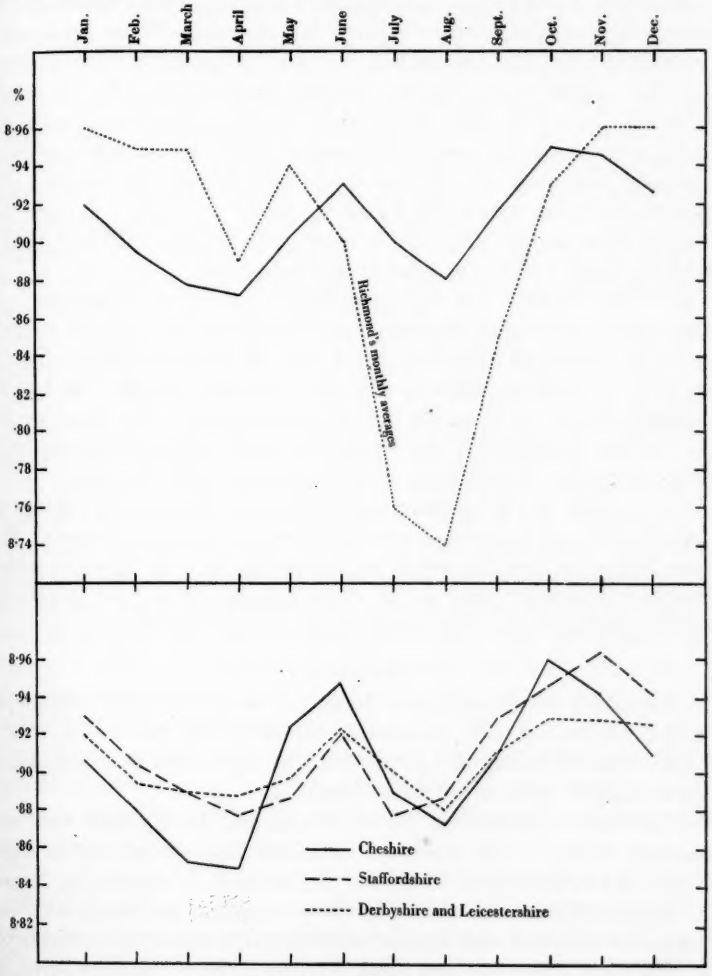
—show a variation of over 0·5 per cent. The district averages (Chart VIII) indicate that the milk from the Cheshire depots varied the most in fat percentage during the year, whilst that of the Derbyshire and Leicestershire milk varied the least. These differences can be explained by variations in management, *i.e.* periods of calving, which factor has been referred to previously.

Charts, which for lack of space have not been reproduced, have been prepared showing the monthly variation in fat percentage for each year of the period. These indicate that August is the month of least yearly variation, in contrast to December, when one may expect rather wide variation from year to year.

Variation in solids-not-fat content.

(1) *Yearly variation* (Charts IX and X).

The average solids-not-fat percentage varied rather less from year to year than the fat percentage, but the difference between the graphs is not very



Solids-not-fat Percentages

Chart XI. Monthly averages for all areas.
Chart XII. Monthly averages for individual areas.

marked. There is a similarity between the solids-not-fat and fat graphs (Charts I and IX), with the exception of 1931, in which year the solids-not-fat graph follows the rainfall graph (Chart III). There certainly appears to be a positive correlation between the yearly average percentage of solids-not-fat and the

annual rainfall data. Examining the district graphs (Chart X), the least variable graph appears to be that of the Derbyshire and Leicestershire district.

(2) *Monthly variation* (Charts XI and XII).

In Chart XI, which illustrates the monthly averages for all areas, is given, for comparison, a graph based on Richmond's monthly average figures (2). There appears to be some disagreement between the two graphs. Whilst both indicate depressions in spring and late summer, the extent of the fall in July–September varies. The comparatively smaller variation shown in the 1923–31 graph is borne out in Chart XII, where the trend of the district graphs is in accordance with the general average graph. The marked fall in Richmond's records for the average percentage of solids-not-fat in July–August may be due, either to the district from which the Aylesbury Dairy Company's supplies were drawn, or to a seasonal difference between periods 1896–1916 and 1923–31. The former appears to be the more likely explanation.

One of the writers (3) has observed that the drying up of pastures in the summer may cause a fall in the percentage of solids-not-fat, and it is suggested that, if the Richmond data were based on milk obtained mainly from the districts in the eastern, southern and south midland counties, the fact that the summer months in these districts are usually much drier than one finds in the Cheshire, Staffordshire and Derbyshire areas, might afford a reasonable explanation for the disagreement of the two graphs under discussion.

As in the case of the fat data, graphs showing the monthly average percentage of solids-not-fat for each year of the period have been prepared. There is some indication that the solids-not-fat percentages are more variable in August and September than in the other months of the year, whilst from October to February the variation from year to year is small.

SUMMARY.

1. Records of the percentages of fat and solids-not-fat in the milk supplies received at twelve depots in the counties of Cheshire, Staffordshire, Derbyshire and Leicestershire during the period 1923–31 have been analysed and the results of analysis given in the form of graphs.

2. The yearly averages for the whole area and for the three sections of the area are illustrated and compared with graphs based upon meteorological data (rainfall and temperature) recorded at stations in, or adjacent to, the area.

3. The monthly average figures for the whole period are shown in the form of graphs and compared with graphs based upon data recorded by Richmond.

4. The data upon which this paper is based indicate a positive correlation between rainfall and average composition of milk for each year.

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(2) —— (1920). *Ibid.*
(3) CRANFIELD (1930). *J. Min. Agric.* **37**, 347–50.

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63. THE MODE OF COMBINATION AND DISTRIBUTION OF TRACES OF HEAVY METALS IN DAIRY PRODUCTS

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Milk as drawn from the cow contains on the average about 0·5 part per million of copper and 2·0 p.p.m. of iron. Additional amounts of these and other metals may occur in milk and its products by solution from surfaces of utensils and plant during processing. In view of the significance of traces of heavy metals nutritionally and on the keeping quality of the fat of a milk product, it seemed justifiable to examine the distribution of the metals between the various fractions of milk obtained during the manufacture of dairy products and to study their mode of combination in these products.

There is considerable evidence that heavy metals form complexes with proteins, the metals entering into an un-ionised condition. Vandevelde (1) studied the interaction between copper salts and milk proteins, and Osborne and Leavenworth (2) examined the copper compounds of some vegetable proteins, while Smythe and Schmidt (3) have investigated iron protein compounds. It was found that proteins were able to bind heavy metal ions, removing almost all ionic metal from solution. In the light of this evidence it is clear that, under conditions of high protein concentration, as in milk, traces of copper and iron would not exist wholly in the ionic condition or in true solution, but that a distribution law greatly in favour of protein-bound metal would be obeyed. Rice and Miscall (4) have stated that when milk contaminated with copper is separated into separated milk and cream, the copper distributes itself between them roughly in proportion to the water. It was concluded that the copper was in solution in the water and that since the copper did not go with the fat, no solution of copper in the fat occurred. This does not preclude the possibility of adsorption of the metal-protein complex on the highly adsorptive fat-globule surfaces of milk products, a point which up to the present has not been studied.

The work described in the present paper consists of (*a*) an examination of the distribution of metal between milk fractions, such as butter and buttermilk from cream, and cream and separated milk from whole milk, and (*b*) a study of the diffusibility of heavy metals through membranes and the potentiometric measurements of their ionic activity under different conditions.

Analytical methods.

The usual routine methods of analysis of dairy products were adopted. The xanthate method was used for the colorimetric determination of copper and the thiocyanate method for the determination of iron. These methods have been described in a previous paper (Davies (5)).

A. THE DISTRIBUTION OF COPPER BETWEEN CREAM AND SEPARATED MILK.

Fresh whole milk was made to contain roughly 13 p.p.m. of copper by adding copper lactate. On analysis, the milk was found to contain 12·95 p.p.m. of copper. One portion (I) (130 c.c.) was centrifuged at 3000 r.p.m. for 15 min. and another portion (II) (250 c.c.) allowed to cream by gravity for 20 hours at 15–18° C. The fat, solids-not-fat and protein nitrogen content of the original milk, and the weights and similar analyses of each layer in I and II were determined. The copper contents of the lower layers of I and II were also determined, that of the cream layers being calculated. Table I gives the results obtained.

Table I. *Distribution of copper in whole milk, between cream and separated milk.*

	I. Centrifuged		II. Skimmed	
	Cream	Separated milk	Cream	Skim milk
Weight of layer (g.)	6·5	128·0	29·2	229·5
Weight of fat (g.)	4·55	Nil	8·45	0·30
Fat in layer (%)	70·0	—	28·9	0·13
Protein nitrogen (mg.)	10	676	132	1187
Water (g.)	1·9	117·4	23·0	206·5
Copper (mg.)	0·07	1·67	0·33	3·02
Copper (p.p.m.)	10·8	13·1	11·3	13·2
$\frac{\text{Copper} \times 10^3}{\text{Protein nitrogen}}$	7·00 (= *A*)	2·47 (= *B*)	2·50 (= *A*)	2·54 (= *B*)
$\frac{\text{Copper} \times 10^5}{\text{Water}}$	3·68	1·42	1·44	1·46
A/*B*	2·80		0·98	

The results given in Table I indicate that with gravity creaming the copper is distributed in direct proportion to the water in the two fractions or in direct proportion to the protein nitrogen. The copper is not distributed according to the weight of fat but the separated layer still contained 0·13 per cent. of fat which possessed a significant area of adsorbing surface. In the centrifuged portion where the residual fat in the separated milk layer was too small to be determined, the copper/protein nitrogen ratio was nearly three times greater in the cream layer. It is clear that adsorption of copper had taken place at the surface of the fat globules. The fat globules remaining in the separated milk from gravity creaming were smaller in size on the average than those in the cream layer and thus possessed a higher adsorptive power relative to their mass than the larger fat globules in the cream. By centrifuging, these smaller globules appeared in the cream layer so that the higher concentration of copper relative to curd nitrogen could be explained. The copper thus tends to distribute itself not according to the weight of fat in any fraction, but according to the effective adsorbing area of the fat globules. But in methods of creaming where a certain fraction of the smaller fat globules remains in the separated milk, the copper distributes itself in proportion to the water or to the protein nitrogen, thus confirming the findings of Rice and Miscall(4).

B. EMULSIFICATION OF BUTTER IN "COPPERED" SEPARATED MILK OF DIFFERENT ACIDITIES.

Series I. Seven 250 g. portions of separated milk containing 50 p.p.m. of copper (as lactate) and 50 g. portions of butter (containing 11·54 per cent. moisture and negligible copper content) were emulsified in an emulsifier made of heavily tinned metal with an electrically driven rotor. The resulting creams were churned in a laboratory churn made of japanned metal with a celluloid lid. The butter samples were washed in the churn and drained free from moisture in cheese cloth, and analysed for copper content and curd nitrogen.

Series II. A similar procedure was adopted in this series, except that 100 g. portions of butter were emulsified in 233 g. of separated milk containing copper as lactate sufficient to make the final cream contain 50 p.p.m. of copper. Six different acidities of milk were used.

Table II gives the results of the analyses.

Table II. *Emulsification of butter in "coppered" separated milk at different acidities and churning. Analyses of butter.*

Exp. No.	Acidity of buttermilk % lactic acid	Copper p.p.m.	Curd nitrogen mg. %	$\frac{\text{Copper} \times 10^3}{\text{Curd nitrogen}}$
		Series I.		
1	0·26	20·6	159	13·0
2	0·36	9·4	71	13·2
3	0·39	12·1	94	12·9
4	0·41	10·7	80	13·4
5	0·49	14·0	113	12·4
6	0·53	9·5	78	12·2
7	0·59	13·4	108	12·4
		Series II.		
1	0·095	14·9	184	8·09
2	0·114	11·5	142	8·11
3	0·166	10·1	156	8·13
4	0·225	14·0	173	8·07
5	0·285	6·8	83	8·17
6	0·395	9·9	123	8·07

From Table II it can be seen that acidity of cream does not affect the amount of copper entering into the butter. The amount of copper in butter made from cream batches of identical copper content but different acidities varied with the curd content (as measured by curd nitrogen) of the butter. (It may be remarked that with the higher acidities (Series I) less copper appeared in the curd, but the results were not significant enough to warrant a general statement to this effect, since curdling of the cream was observed to occur in Exps. 5, 6 and 7.)

C. Butter from "coppered" cream. Distribution of copper between butter and buttermilk.

Twenty pounds of cream of known analysis (30·8 per cent. fat) were "coppered" to the extent of 25 p.p.m. After division into four batches, the following types of butter were prepared:

I. Churned at once (acidity 0·18 per cent.).

II. The cream was acidified with lactic acid to 0·5 per cent. and churned.

III. The cream was allowed to ripen to 0·5 per cent. acidity and churned.

IV. The cream was neutralised to an acidity of 0·08 per cent. by the addition of the calculated quantity of sodium bicarbonate, and after allowing to stand for 16 hours, churned.

The butter samples were weighed and analysed for curd and copper content and the distribution of these constituents determined (Table III).

Table III. *Distribution of copper between butter and buttermilk from cream at various acidities.*

			Butter				Buttermilk			
Batch	Butter lb.	Butter-milk lb.	Moisture %	Curd N mg. %	Cu p.p.m.	A $\frac{Cu \times 10^3}{Curd\ N}$	Total curd N g.	Total Cu mg.	B $\frac{Cu \times 10^3}{Curd\ N}$	A/B
I	1·80	3·20	11·20	53·0	7·0	13·2	7·738	51·0	6·6	2·0
II	1·80	3·20	12·20	56·3	7·5	13·2	7·708	50·6	6·6	2·0
III	1·87	3·13	12·20	77·4	7·9	10·1	7·508	50·0	6·5	1·6
IV	1·76	3·24	11·18	53·3	8·5	15·9	7·826	51·4	6·6	2·4

From Table III it can be seen that the copper associated with unit weight of curd in butter is roughly twice that associated with the protein of the buttermilk. This can only be explained by assuming adsorption of the copper proteinate at the fat-globule surface with the result that a greater concentration of copper relative to curd nitrogen adheres to the churned butter as curd than is shown by unadsorbed proteinate in the buttermilk and washings. The results also show that acidity of cream has an effect on the degree of adsorption. Butter from cream neutralised to a low acidity shows the highest degree of adsorption, while butter from very acid cream, where the acidity has taken the normal period of time for development, shows the lowest degree of adsorption. Butter from cream of normal acidity, or from cream acidified artificially and churned at once, does not differ as regards the degree of adsorption shown.

The *copper/curd nitrogen* ratios for the butters do not show the same constancy as in Table II. This may be due to their lower curd contents, which would tend to enhance differences which in Table I did not appear to be significant. The *copper/curd nitrogen* ratios vary with the acidity of the cream, the butter from neutralised cream showing the highest ratio, while the normally ripened cream butter showed the lowest ratio. Acidity of the cream evidently decreases the concentration of the proteinate associated with

the fat globules and tends to cause more ionic copper to be present, a statement later borne out in the diffusibility experiments.

In order to study further the distribution of copper between butter and buttermilk, 0·7 lb. of butter II (containing 7·6 p.p.m. of copper) was emulsified with 2 lb. of separated milk (containing 0·22 p.p.m. of copper). The cream (1·226 kg.) contained 2·62 mg. of copper and on churning gave 0·63 lb. of butter (2·07 lb. of buttermilk). The analyses of both fractions are given in Table IV.

Table IV. *Butter II reconstituted with separated milk and churned. Analyses of butter and buttermilk.*

	Weight g.	Cu mg.	Cu p.p.m.	Curd N mg.	$\frac{\text{Cu} \times 10^3}{\text{Curd N}}$	A/B
Butter	286	0·71	2·49	111	$6{\cdot}40 = A$	1·35
Buttermilk	940	1·91	2·03	403	$4{\cdot}74 = B$	

(Acidity of buttermilk = 0·24 %.)

Although the resulting butter was higher in curd than butter II, the *copper/curd nitrogen* ratio was appreciably in favour of the butter, which showed that part of the copper proteinate adheres to the fat-globule surface in spite of alternate inversions of the type of emulsion.

D. Emulsification experiments with "ironed" separated milk. The effect of added lecithin.

I. Butter (225 g.) was emulsified with 575 g. separated milk after the addition of ferrous lactate solution sufficient to give a concentration of 30 p.p.m. of iron in the cream.

II. Butter (168 g.), 1·5 g. B.D.H. egg lecithin and 432 g. of separated milk were emulsified together after the addition of ferrous lactate sufficient to give 30 p.p.m. in the cream.

Both batches of cream were churned and the butter analysed for curd and iron (Table V).

Table V. *Emulsification experiment using iron salts.*

		Butter				Buttermilk			
	Weight g.	Curd N mg. %	Fe p.p.m.	Fe mg.	A: $\frac{\text{Fe} \times 10^3}{\text{Curd N}}$	Curd N mg. %	Fe mg.	B: $\frac{\text{Fe} \times 10^3}{\text{Curd N}}$	A/B
I	214	117	17·1	3·66	14·6	317	21·60	11·6	1·26
II	106	112	12·9	1·37	11·5	303	17·57	9·6	1·20

I = butter, separated milk, iron 30 p.p.m.
II = butter, separated milk, egg lecithin, iron 30 p.p.m.

Table V demonstrates a greater *iron/curd nitrogen* ratio in both types of butter studied, showing adsorption of the iron proteinate at the fat-globule surface.

From the one set of results given in Table V illustrating the effects of lecithin, it may be gathered that adsorption of the metal proteinate is de-

pressed by added lecithin (compare values for A). This is to be expected from the hydrophilic properties of lecithin, in that the surface tension of the fat globules would be decreased, thus lowering the adsorptive capacity of the surface. The resulting effect on the iron content of the butter is that less iron is associated with the curd than with the butter made from cream to which no lecithin was added.

E. The entry of heavy metal into the fat phase under different conditions of acidity.

This investigation was carried out in order to study the effect of the normal range of acidity on the entry of copper into the fat phase from aqueous solution, and the part played by milk protein under such conditions.

(*a*) *In the absence of milk protein.*

Seventy-five gram portions of olive oil in stoppered bottles were mixed with 20 c.c. of copper lactate solution containing 0·0375 per cent. of copper. The copper lactate solutions were made to cover a range of acidity of from nil to 2·0 *N* lactic acid by the addition of known amounts of 10 *N* lactic acid solution. The bottles were kept at 38° C. for 14 days with frequent shaking. Some of the oil was separated by filtration at the end of the period and copper determined in 25 g. portions.

Table VI gives the results obtained.

Table VI. *Entry of copper into olive oil in contact with aqueous copper solutions at different acidities* (14 *days*).

Acidity (normalities)	0·0	0·1	0·5	1·0	2·0
Copper (p.p.m.)	3·59	2·76	2·04	1·89	1·27

Maximum solution of copper by the fat occurred at neutrality, and the degree of solution fell off with increasing acidity.

(*b*) *The effect of milk protein.*

Twenty-five c.c. of separated milk were added to the contents of the bottles (see (*a*)) and thoroughly shaken. The bottles were kept at 38° C. for 24 hours and frequently shaken. Gravity creaming was allowed to occur for 16 hours at room temperature (16° C.) and the "cream" in each sample analysed for curd nitrogen and copper. Some pure oil was separated from portions of the cream and analysed for copper content.

Table VII shows the results obtained.

On studying Table VII it can be seen that an original acidity from nil to 0·5 per cent. did not appreciably alter the *copper/curd nitrogen* ratio when olive oil was creamed in the presence of milk protein. But slight gains in the copper contents of the oil itself were observed. This was possibly due to the increased concentration of copper maintained by the protein films on the

Table VII. *Olive oil, copper lactate and varying amounts of lactic acid. Copper and curd nitrogen contents* (40 *hours*).

Sample	Original acidity (normalities)	Curd N p.p.m.	Cu p.p.m.	$\frac{Cu \times 10^3}{Curd\ N}$	Cu in oil p.p.m.	Gain in Cu content of oil p.p.m.
1	0·0	355	4·92	13·9	3·60	0·0
2	0·1	1371	17·00	12·4	3·00	0·24
3	0·5	1309	15·50	11·8	2·10	0·06
4	1·0*	353	5·30	14·4	2·00	0·11
5	2·0*	277	6·20	22·4	1·40	0·13

* Precipitation of the curd had occurred in samples 4 and 5.

oil-globule surface and a lower acidity due to the buffering effect of the milk. There was no tendency for copper to enter the oil at a greater rate at the higher acidities. The higher acidities (4 and 5) precipitated the curd, but an amount of curd nitrogen about equal to that on the sweet milk (1) was still left on the oil-globule surfaces. In this case the "cream" consisted of solid curd entrapped by the oil globules.

F. The diffusibility of heavy metal ions in milk at different acidities.

This series of experiments was carried out to obtain a comparative idea of the amount of truly soluble metal under different conditions. Collodion capsules were used as membranes, and these, containing 25 c.c. of milk of known acidity and copper or iron content, were suspended in distilled water (300 c.c.). The length of time for diffusion was 24 hours, after which period the water was weighed and its copper or iron content determined. The range of acidities covered up to 1 per cent. lactic acid, and the copper and iron contents (including the "natural" contents) were 100 p.p.m.

It must be pointed out that as soon as ionic metal diffused from the milk the equilibrium between ionic metal and metal proteinate in the milk tended to be disturbed. The equilibrium would be maintained by bound metal going to the ionic form. What was measured by a 24 hours' diffusion was not the absolute state of the metal in the milk, but the cumulative effect of the above reaction, and, since the ratio of bound to ionic metal may be considered constant for any one condition of the milk, the results for the different acidities may be looked on as comparative.

Table VIII gives the results obtained.

Table VIII. *Diffusibility of copper and iron* (100 *p.p.m.*) *in milk at different acidities* (24 *hours' diffusion*).

Acidity % lactic acid	*p*H	Copper				Iron		
		Total Cu mg.	Diffusible Cu mg.	% diffusible	Acidity of dialysate as % lactic acid	Total Fe mg.	Diffusible Fe mg.	% Fe diffusible
0·18	6·6	2·50	0·14	5·6	—	2·50	0·10	4·0
0·27	6·1	2·50	0·21	8·4	0·019	2·50	0·15	6·0
0·40	5·6	2·50	0·37	14·8	0·032	2·50	0·31	12·4
0·60	4·9	2·50	0·65	26·0	0·041	2·50	0·58	23·2
1·00	4·7	2·50	1·08	43·2	0·071	2·50	0·93	28·2

A study of Table VIII reveals that the amount of diffusible heavy metal increased directly with the acidity. From sweet milk, about 1/18th of the copper and 1/25th of the iron were diffusible in 24 hours, whilst at 0·40 per cent. acidity about 1/7th of the copper and 1/8th of the iron were diffusible. The rate of diffusion of iron was about 0·8 that of copper.

An attempt was made to correlate the diffusion of heavy metals with that of hydrogen ions from the milk. Owing to the low acidity of the diffusates from low acidity milk this could not be gauged for these samples, but the titratable acidity of the diffusates from high acidity milk was appreciable and the amounts of lactic acid found varied directly with the milk acidity and with the amounts of diffused metal.

Thus it was established that in high acidity conditions in milk there was more metal in the ionic form than in sweet milk, that is, the equilibrium between bound and ionic metal in milk is disturbed towards the ionic form by increase in acidity.

G. Attempts at determining the concentration of the ionic forms of heavy metals in milk by potentiometric measurements.

This investigation was carried out in order to determine the potentials which the copper in small concentrations in milk would give against a copper electrode using copper in contact with very dilute copper sulphate as the other half-cell. The copper electrodes used were made from stout wire on which a fresh plating was deposited from $N/50$ copper sulphate slightly acidified with nitric acid. The electrodes were tested against one another in the same solution and were found to behave normally. A Tansley potentiometer and mirror galvanometer were used for the potential measurements. The "known" half-cell was 0·0001 N copper sulphate, the other half-cell being milk containing varying amounts of copper added as copper sulphate. Table IX gives examples of the results obtained.

Table IX. *Concentration of ionic copper in milk at different acidities and copper contents.*

Copper content of milk Normality	Acidity of milk (% lactic acid)	*p*H	Concentration of ionic copper	Fraction of copper ionised
1×10^{-4}	0·17	6·6	$1{\cdot}26 \times 10^{-10}$	$1{\cdot}26 \times 10^{-6}$
1×10^{-3}	0·17	6·6	$7{\cdot}94 \times 10^{-8}$	$79{\cdot}4 \times 10^{-6}$
1×10^{-3}	0·25	6·3	$9{\cdot}12 \times 10^{-8}$	$91{\cdot}2 \times 10^{-6}$
1×10^{-3}	0·50	5·4	$1{\cdot}59 \times 10^{-7}$	$159{\cdot}0 \times 10^{-6}$

It can be deduced that the amount of heavy metal in the ionic form at the acidity range commonly met with in milk is of low order of magnitude, and that under conditions of increase in acidity there is an increase in the amount of ionic metal.

The addition of milk to a copper solution.

The effect of the addition of small quantities of milk to a copper solution of known strength and varying *p*H was also investigated. Table X gives the results obtained with a 0·01 *N* solution of copper sulphate.

Table X. *The effect of the addition of milk to a* 0·01 *N copper sulphate solution at different* p*H. 50 c.c.* 0·01 *N copper sulphate in each electrode vessel.*

Total vol. c.c.	Vol. added	*p*H	Voltage	Ionic concentration of copper. Fraction ionised
50	—	5·7	0·000	1×10^{-2}
50	1 drop milk	5·7	0·019	$2{\cdot}2 \times 10^{-3}$
50·1	4 drops milk	5·7	0·026	$1{\cdot}3 \times 10^{-3}$
51·1	1 c.c. milk	5·7	0·029	$1{\cdot}0 \times 10^{-3}$
52·1	1 c.c. milk	5·7	0·033	$7{\cdot}2 \times 10^{-4}$
54·1	2 c.c. 0·1 *N* alk.	5·8	0·054	$1{\cdot}6 \times 10^{-4}$
55·1	1 c.c. 0·1 *N* alk.	5·8	0·090	$7{\cdot}9 \times 10^{-6}$
57·1	2 c.c. 0·1 *N* alk.	>6·8	0·202	$1{\cdot}1 \times 10^{-9}$
58·1	1 c.c. 0·1 *N* HCl	6·7	0·142	$1{\cdot}3 \times 10^{-7}$
58·6	0·5 c.c. 0·1 *N* HCl	6·7	0·103	$2{\cdot}8 \times 10^{-6}$
59·1	0·5 c.c. 0·1 *N* HCl	6·0	0·089	$7{\cdot}2 \times 10^{-6}$
61·1	2·0 c.c. 0·1 *N* HCl	4·4	0·061	$7{\cdot}9 \times 10^{-5}$
62·1	1·0 c.c. 0·1 *N* HCl	<3·6	0·045	$2{\cdot}8 \times 10^{-4}$

From Table X it can be observed that the addition of a small amount of milk protein causes a large amount of ionic metal to form compounds having a low degree of dissociation. By changing the *p*H in the alkaline direction this power per unit weight of protein is increased until the amount of ionised metal is very small. Increasing the acidity again brings more ionic copper into solution, but under conditions of high acidity in the presence of milk protein, the state of complete ionisation was not recovered.

SUMMARY.

Small amounts of heavy metal are distributed between cream and separated milk in proportion to the curd nitrogen, but on completely centrifuging the fat a higher concentration of metal occurs in the cream, showing adsorption of complex proteinate at the fat-globule surface.

The heavy metal entering into butter from cream of different acidities is not affected by the acidity but by the curd-nitrogen content of the butter, but the copper associated with the curd in butter is roughly twice that associated with the protein nitrogen of the buttermilk. This is explained by adsorption of heavy metal on the fat-globule surface, which holds alike in water in oil as well as oil in water emulsion.

The entry of heavy metal into the fat phase in the absence of protein is greatest at neutrality but decreases with increasing acidity. The presence of protein increases slightly the amount of heavy metal entering into the fat owing to adsorptive and buffering effects of the protein at the oil-water interface in the emulsion.

The amount of diffusible heavy metal increases directly with the acidity and runs parallel with the diffusion of hydrogen ions.

The concentration of the ionic form of heavy metal in milk is of a low order, but increase in acidity causes more ionic metal to form. Evidence from potentiometric measurements in milk containing known amounts of heavy metal, or from the addition of milk to a standard copper solution at various acidities, confirms this finding.

The author is indebted to Miss D. V. Dearden for preparing the samples of butter.

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64. FACTORS AFFECTING THE SOLUBILITY OF MILK POWDERS

II. THE INFLUENCE OF TEMPERATURE OF RECONSTITUTION ON PROTEIN SOLUBILITY.

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(With 6 Figures.)

In the previous paper (1) it was shown that the heat treatment to which a milk powder is subjected during manufacture may result in the production of two distinct types of protein insolubility, namely insolubility due to denaturation of the protein while still in solution, and insolubility caused by over-heating the milk powder while in a dry state. The insolubility due to denaturation was shown to be independent of the temperature of reconstitution of the milk powder. On the other hand the insolubility due to dry heating was found to vary with the temperature of reconstitution, the protein being relatively insoluble when reconstituted in water held at 20° C., but completely soluble when mechanically stirred for 30 min. in water held at 90° C. It was suggested that this latter type of insolubility might be due to the removal of water of imbibition from the protein particles, since such an explanation would account for the effect of hot water in restoring the solubility of the protein. In the previous paper, however, observations on the effect of temperature of reconstitution were limited to the two extreme temperatures, *i.e.* 20 and 90° C.

The methods used for the determination of the solubility of milk powders vary widely as regards both technique and temperature of reconstitution. Marquardt (2) recommends treating the sample with successive charges of water at 50–55° C., centrifuging after each extraction, and determining the amount of insoluble residue. Supplee (3) reconstitutes the milk powder by stirring for 10 min. in water held at 65° C., and subsequently determines the total protein in the supernatent liquid after centrifuging. On the other hand, Hunziker (4) records results obtained by reconstituting for 10 min. at 25° C. and for 5 min. at 100° C. respectively. At the time when these methods were devised, however, the existence of two types of protein insolubility in milk powders was not clearly recognised. In view of the increased knowledge now available and of the important bearing of this knowledge on the technique of solubility determination, it appeared desirable to investigate in more detail the influence of temperature of reconstitution on the solubility of milk powders.

Technique.

Throughout the present investigation the technique employed for the determination of protein solubility has been identical with that noted in the previous paper (1).

Results and discussion.

The influence of temperature of reconstitution on the solubility of an artificially heated milk powder.

It appeared probable that any alterations produced in the solubility of milk powders by variations in the temperature of reconstitution would be

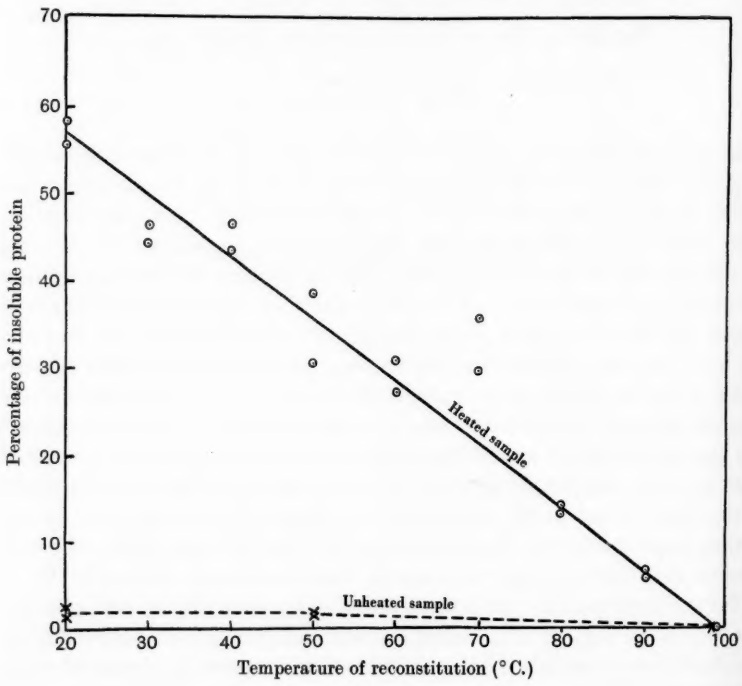

Fig. 1.

primarily dependent on the effect of the dry heat treatment to which the powder had been subjected during manufacture. Preliminary experiments were therefore limited to a study of this aspect of solubility.

A quantity of spray-dried separated milk powder was heated in thin layers in an electric air oven for 6 hours at 105–110° C. The percentage of insoluble protein in the heated powder, as well as in the original (unheated) powder, was then determined after reconstitution at various temperatures between 20 and 100° C. The results are shown in Fig. 1. It will be seen that the solubility of the unheated sample varied but slightly with temperature of reconstitution,

the percentage of insoluble protein falling from under 2 per cent. at 20° C. to nil at 99° C. On the other hand the sample which had been subjected to dry heating showed practically 60 per cent. of protein to be insoluble when the powder was reconstituted at 20° C., but the solubility increased progressively with increasing temperature of reconstitution, until at 100° C. the whole of the protein was soluble.

It was found impossible to obtain close agreement between duplicate determinations on this powder, probably owing to difficulties in sampling, and considerable errors were undoubtedly involved in determining the solubility at some temperatures (see especially the results for 70° C.). Taken as a whole, however, the results indicate that, with a constant time of heating, the percentage of protein rendered soluble is directly proportional to the temperature of reconstitution, as shown by the straight line relationship illustrated in Fig. 1.

The influence of temperature of reconstitution on the solubility of commercially prepared milk powders.

The above results were obtained on an artificially heated milk powder. The next step was to investigate the influence of temperature of reconstitution on the solubility of commercial milk powders. For this purpose the percentage of insoluble protein in each of the following eight samples was determined after reconstitution at various temperatures between 20° and 100° C.:

Sample I. A commercial brand of roller-dried separated milk powder.

Sample II. A commercial brand of roller-dried whole milk powder containing added lactose.

Sample III. A sample of dried separated milk powder prepared on a single-cylinder model roller drier: steam pressure in roll 25 lb./in.^{2}, time of drying 1 sec.

Sample IV. A sample prepared on the same plant, but from whole milk (4·2 per cent. butterfat).

Sample V. Identical with IV, but prepared from separated milk (0·1 per cent. butterfat).

Sample VI. Identical with IV, but prepared from whole milk to which cream had been added (7·7 per cent. butterfat).

Sample VII. A commercial brand of spray-dried separated milk powder.

Sample VIII. A commercial brand of spray-dried whole milk powder.

The results of the determinations on these samples are shown in Figs. 2–6. For convenience they will be discussed under three heads:

(*a*) There is an increase in the solubility of each roller-dried sample between 20 and 50° C. There is little doubt that this increase is chiefly due to the fact that the milk powders contain a considerable proportion of protein which has been rendered insoluble by dry heating, but which (as shown in the preceding section) will regain its solubility when reconstituted in hot water. With regard to the whole milk powders, however, the increased solu-

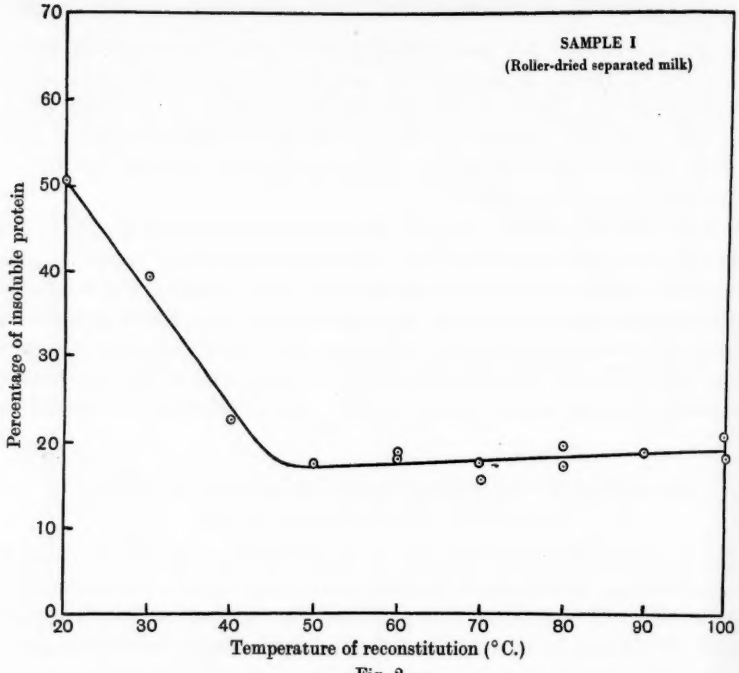
SAMPLE I
(Roller-dried separated milk)
Percentage of insoluble protein
0
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100
Temperature of reconstitution (° C.)

Fig. 2.

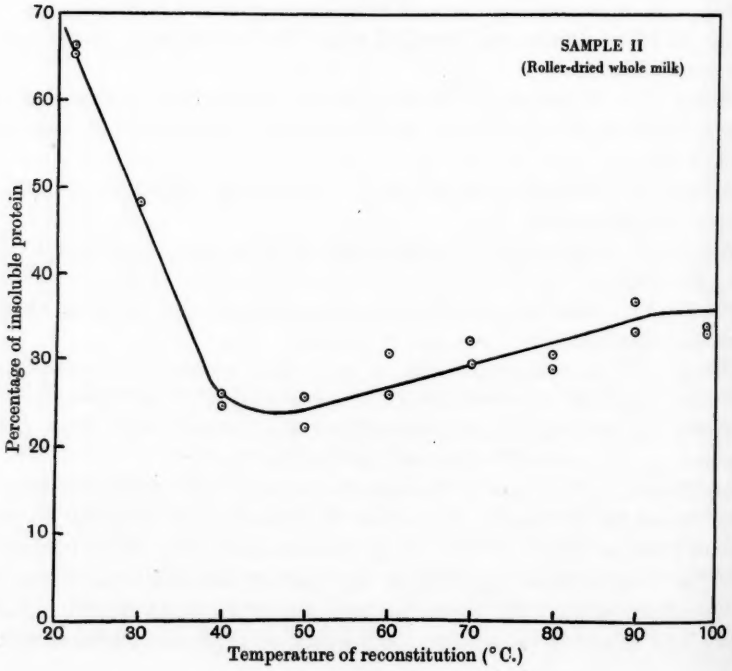
SAMPLE II
(Roller-dried whole milk)
Percentage of insoluble protein
0
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100
Temperature of reconstitution (° C.)

Fig. 3.

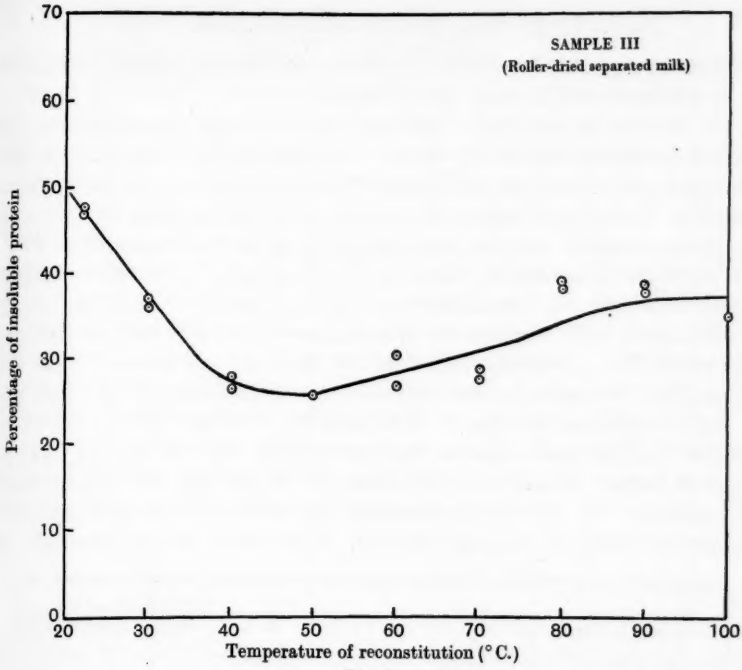
SAMPLE III
(Roller-dried separated milk)
Percentage of insoluble protein
Temperature of reconstitution (° C.)
0
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100

Fig. 4.

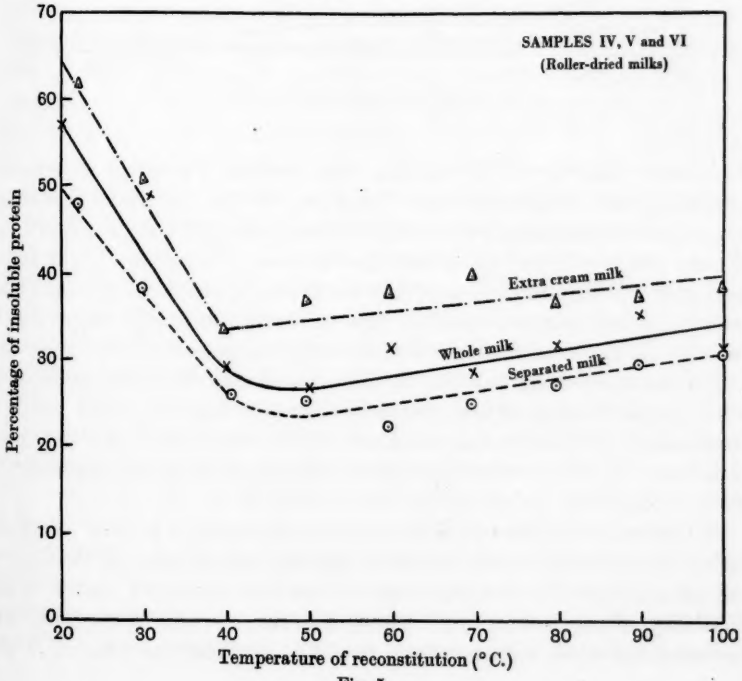
SAMPLES IV, V and VI
(Roller-dried milks)
Extra cream milk
Whole milk
Separated milk
Percentage of insoluble protein
Temperature of reconstitution (° C.)
0
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100

Fig. 5.

bility at the higher temperatures may also be attributed in part to the influence of the fat content of the milk (see (*c*) below).

(*b*) Between 50 and 100° C. the roller-dried samples show a more or less marked decrease in solubility, which is best illustrated in Figs. 3 and 4. This decreased solubility might very naturally be attributed to the precipitation of albumin by the prolonged reconstitution at the higher temperatures, since Rupp (5) has shown that the denaturation of albumin commences at 63° C. and is completed instantaneously at 85° C. Further, in the samples examined the increase in protein insolubility is about 10 per cent., while the percentage of albumin in milk accounts for approximately 12·5 per cent. of the total nitrogen (6). The experimental facts do not, however, appear to bear out this assumption. Separate determinations of casein, albumin *plus* globulin, and non-protein nitrogen were made on Sample III after reconstitution at 20, 50 and 100° C. The results showed that between 50 and 100° C. there was an apparent increase in insolubility of casein of 7·80 per cent., while the albumin *plus* globulin only showed an increased insolubility of 3·68 per cent. The non-protein nitrogen remained constant. Such results may be partly due to

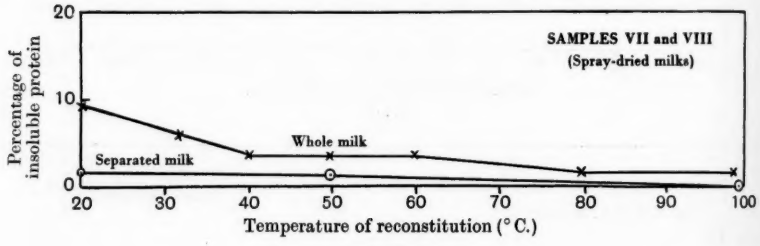

Fig. 6.

the inherent difficulty of determining these proteins separately after reconstitution at high temperatures (see Wright (1)). On the other hand there may be a physical association between the colloidal properties of the casein and albumin which will lead to mutual precipitation. In support of the latter suggestion it is significant to note that the two spray-dried samples examined do not show any decreased solubility after reconstitution at high temperatures, and that the casein of these powders is almost completely soluble. That is to say, in the spray-dried powders the high percentage of soluble casein may induce *mutual stability* of both proteins, whereas in the roller-dried powders, which contain far less soluble casein and in which the casein may have been "sensitised" by the previous heat treatment during drying, the denaturation of the albumin may induce *mutual precipitation*.

(*c*) In order to differentiate between the direct effect of heat treatment during drying on the protein solubility, and the indirect effect of the fat content on solubility (for instance, through the influence of the latter on the miscibility of the powder with water), experiments were made with both separated and whole milk powders. It will be seen that the effect of the fat

is to depress protein solubility throughout the entire range of temperatures at which the samples were reconstituted. This is best shown in Fig. 5, where the solubilities of three powders, all derived from the same bulk of whole milk and all manufactured under similar conditions, are compared. It is also indicated in the comparative solubilities of the two commercially prepared roller-dried samples (I and II) and of the two spray-dried samples (VII and VIII), although direct comparisons are not valid in these instances, the powders being manufactured on different types of plant. From the point of view of the present study, however, the important fact is that the influence of fat content on protein solubility is approximately constant throughout the entire range of temperatures of reconstitution: that is to say, the presence of the fat in the powder does not invalidate any conclusions which may be drawn from the comparative solubilities of the protein at different temperatures. The increase in solubility between 20 and 50° C., and the subsequent decrease between 50 and 100° C., occur whether the milk powder is manufactured from separated milk or from whole milk.

Application of the results to the technique of solubility determination.

The above results were obtained by the use of a technique far more drastic than that normally employed in routine solubility determinations, *i.e.* the samples were reconstituted in 10 per cent. suspension with high-speed stirring for 30 min., centrifuged at 3000 R.P.M. for 15 min., and the protein determined in the resulting supernatant liquid. It should be clearly understood, therefore, that any conclusions which are drawn from these results cannot be indiscriminately applied to routine methods of solubility determination which are based on different times and conditions of reconstitution. The conclusions are only of value in directing attention to the chief factors which must be considered in modifying existing technique or in formulating revised methods. Subject to this qualification, the following comments may be made:

First, the results show that the temperature of reconstitution is an extremely important factor in determining the solubility of a milk powder. Thus, if the results for sample II are examined, it will be seen that the extreme differences in protein insolubility range from 70 per cent. when the milk powder is reconstituted at 20° C., to 25 per cent. when it is reconstituted at 50° C., while at 100° C. the value lies between 30 and 40 per cent.

Second, it is possible in the light of previous work and of the present investigation to give an explanation of these observed variations in solubility. Thus it appears that the increase in solubility between 20 and 50° C., is due to the presence of protein which has been rendered insoluble by over-heating in a dry state, the proportion of such protein in sample II being, for example, about 70 per cent. *minus* 25 per cent., or 45 per cent. of the total protein in the powder. On the other hand, the protein which remains insoluble at 50° C., *i.e.* 25 per cent., is undoubtedly that fraction which has been completely

denatured by moist heating during the drying process and which is therefore irreversibly coagulated.

Third, the decreased solubility which occurs between 50 and 100° C., which appears to be the result of the heat treatment during reconstitution, demonstrates that, in selecting a temperature of reconstitution for a routine method, it is essential to avoid temperatures higher than about 60° C.

Fourth, the results of the determinations made on whole milk powders indicate that the fat content of the powder will not normally interfere with the general nature of the results.

In view of these facts it is apparent that, using the technique employed in the present investigation, the most valuable indication of the extent and nature of the protein insolubility will be obtained by reconstituting a milk powder at the two temperatures, 20 and 50° C., since the values obtained at these temperatures will enable the observer to gauge the extent of protein insolubility induced during manufacture by moist and by dry heating respectively, and will thus indicate any modifications which may be necessary in the control of the thermal conditions in his plant. If less drastic methods of reconstitution are employed, it might be necessary to raise the latter temperature somewhat, but it is doubtful whether a temperature in excess of 60° C. could be employed without serious risk of denaturing the albumin.

ACKNOWLEDGMENTS.

Our thanks are due to Mr James Kirkwood, Director of Practical Dairying at the West of Scotland Agricultural College, for his kind assistance in preparing certain special samples of milk powder used in this work. One of us (G.R.H.) is indebted to the Agricultural Research Council for the award of a Research Scholarship during the tenure of which this work was carried out.

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65. THE COMPOSITION OF THE "ISOTONIC DILUENT" IN SAMPLES OF MILK LOW IN SOLIDS-NOT-FAT

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During investigations spreading over the past three years in these laboratories on the problem of milk low in non-fatty solids, considerable attention has been paid to the constituents responsible for the deficiency. Daily analysis of typical milk from a herd of Shorthorn cows over a period of over three years (Golding, Mackintosh and Mattick (6)), has also added to our knowledge of what the composition of *normal* milk produced under typical conditions of commercial management should be. During the winter of 1930 it was found that a low lactose content, which was approximately gauged from the chloride content, was mostly responsible for low solids-not-fat. A widening of the scope of the investigation in the winter of 1931 afforded information concerning the amount and distribution of the protein in samples of normal milk compared with those in samples of varying deficiency in solids-not-fat (Davies (4)). In all cases a lower casein content was found in the latter samples. With this were associated larger and more variable amounts of albumin and globulin and a higher non-protein nitrogen content. The idea was therefore conceived that the secretion from cows giving milk low in solids-not-fat contained a diluent poor as regards its content of compounds of high molecular weight and calorific value. An analysis of the problem from this point of view was therefore considered profitable.

The casein/nitrogen relationship in normal milk.

One of the main conclusions arrived at in a previous paper by the author (Davies (4)) was that, in normal samples of milk, casein nitrogen accounted approximately for 76 per cent. of the total nitrogen. Analysis of the results for total and casein nitrogen for 148 samples of morning's milk from a typical herd of Shorthorn cows (Golding *et al.* (6)) further showed that the casein nitrogen accounted for 76·5 (S.D. 2·16) per cent. of the total nitrogen. The results for 61 other samples of similar milk analysed by the author (unpublished work) have shown that casein nitrogen has accounted for 76·7 (S.D. 1·09) per cent. of the total nitrogen in the samples. The total protein nitrogen in the last samples also on the average has accounted for approximately 94 per cent., albumin nitrogen 12 per cent., and globulin nitrogen 6 per cent. of the total nitrogen.

Definition of the "isotonic diluent."

With regard to the samples of milk deficient in solids-not-fat it has been observed (*a*) that they showed invariably the same depression of freezing-point as that of samples of normal milk and hence possessed the same osmotic pressure (unpublished data) and (*b*) that the percentage of the total nitrogen as casein nitrogen fell below the 76 per cent. level by an amount varying approximately with the deficiency of solids-not-fat in the samples. This decrease in percentage of casein nitrogen was counterbalanced by increases in other protein (albumin and globulin) nitrogen and non-protein nitrogen. In other words, the casein appeared to be diluted with a liquid containing certain concentrations of compounds containing other nitrogen groups. It was therefore conceivable that the samples of milk as analysed could be taken as being made up of two fractions, (*a*) a *true-milk fraction* and (*b*) a *diluting fraction*. The true-milk fraction would contain all the casein nitrogen and its total nitrogen (100/76 times the casein nitrogen) would be made up of the various quotas of albumin, globulin and non-protein nitrogen in amounts corresponding to their average distribution in *normal* milk. The residual nitrogen of each group calculated by difference from the analytical figures for each sample would be appropriated by the diluting fraction, containing, of course, no casein nitrogen.

It can be assumed that the true-milk fraction would possess the same osmotic pressure as normal milk, and hence the diluting fraction would also possess the same osmotic pressure. The diluting fraction, therefore, could be termed the "isotonic diluent" of the true-milk fraction, the mixture constituting the sample as analysed. The amount of true-milk fraction could be calculated from the percentage of total nitrogen as casein nitrogen divided by 76. Thus, for a sample showing 65 per cent. of total nitrogen as casein nitrogen the percentage of "true milk" would be 85·5 and that of the "isotonic diluent" 16·4.

Calculating the composition of the "isotonic diluent."

With all the nitrogen data available from the analysis, it was considered worth while to calculate the percentage composition of the isotonic diluent for as many samples as possible. Table I illustrates the method of calculation for one sample.

Table I. *Method of calculating the composition of the isotonic diluent.*

Sample: Alison 17. v. 32 a.m. Dilution $= 100 - \frac{63{\cdot}3}{76} = 16{\cdot}7$ %.

	Whole sample %	True-milk fraction g.	Residual in diluent (16·7 %) g.	Diluent %
Total N	0·4577	0·3814	0·0763	0·457
Protein N	0·4030	0·3584	0·0446	0·267
Casein N	0·2898	0·2898	—	—
Albumin N	0·0861	0·0460	0·0401	0·240
Globulin N	0·0271	0·0230	0·0041	0·025
Non-protein N	0·0547	0·0230	0·0317	0·190

This method of calculation was applied to the analytical data of 38 samples; the results being given in Table II. The average of the total nitrogen values with standard deviation was calculated. The range of nitrogen distribution in normal blood serum is also included in the table. To economise space the nitrogen distributions of the analysed milk samples have been omitted. The average distributions for the samples from individual cows are given in Table II, and those for "Bulk (*b*)" in Table I of the author's previous paper (Davies (4)).

INTERPRETATIONS OF THE PERCENTAGE OF CHLORINE IN THE MILK SAMPLES.

There were not sufficient data available in the analyses for the calculation of the chlorine content of the diluent. The chlorine content of each milk sample had been determined, from which it was possible only to calculate the approximate lactose content of each sample and hence of the true-milk fraction by using the relationship between lactose and chlorine content suggested by Mathieu and Ferré (7) (lactose (per cent.) = 7–19·6 × Cl per cent.) and which had been proved by the author (Davies (5)) to hold for samples low in solids-not-fat. In most cases, the lactose in the true-milk fraction amounted to that found present in normal milk (*i.e.* approximately 5 per cent.) which served as an approximate check on the figures given for the amount of isotonic diluent in each sample.

The chlorine figures of the samples were plotted against the dilution. A straight line represented by the equation $D = 3{\cdot}8\ \mathrm{Cl} - 0{\cdot}37$ roughly covered the points (D = dilution fraction, Cl = percentage of chlorine) which for 100 per cent. dilution gave a value 0·360 per cent. for chlorine. This approximated closely the chlorine content of a hypothetical lactose-free milk calculated from the above equation of Mathieu and Ferré (0·357 per cent.). It seemed therefore that the chlorine content of the diluent approached that of a liquid isotonic with milk, but in which the lactose was replaced by its isotonic equivalent of ionised chlorides. The value was 16 per cent. higher than the maximum value of chlorides, in blood serum (0·303 per cent.).

DISCUSSION OF RESULTS.

It can be observed from an examination of the results that, irrespective of the amount of isotonic diluent in each sample, the content of total nitrogen was fairly regular (0·458 (S.D. 0·036) per cent.) and falls within the range of total nitrogen in blood serum and incidentally within the range of total nitrogen of the milk samples analysed. The total protein nitrogen, on the other hand, showed a much greater variation, whilst the albumin and globulin distributions did not conform to any uniform generalisation. The non-protein nitrogen was also irregular, being in most samples from three to five times its value in blood serum.

Table II. *Composition of isotonic diluent.*

Cow ...	A						B				C			D					X				
	19. iv. 32*a*	3. v. 32*a*	20. iii. 32*a*	20. iii. 32*b*	17. v. 32*a*	5. iv. 32*a*	19. iv. 32*a*	20. iii. 32*a*	20. iii. 32*b*	5. iv. 32*a*	19. iv. 32*a*	17. v. 32*a*	31. v. 32*a*	19. iv. 32*a*	3. v. 32*a*	17. v. 32*a*	31. v. 32*a*	14. vi. 32*a*	26. iv. 32*a*	10. v. 32*a*	24. v. 32*a*	7. vi. 32*a*	21. vi. 32*a*
Dilution %	50·4	28·3	25·3	35·1	24·4	31·2	28·3	20·9	18·0	26·5	13·0	15·2	16·2	21·2	17·6	16·7	19·6	21·2	12·9	6·4	15·3	8·4	6·3
%																							
Total N	0·411	0·414	0·429	0·473	0·421	0·445	0·430	0·482	0·437	0·457	0·499	0·501	0·524	0·421	0·462	0·457	0·451	0·460	0·429	0·436	0·443	0·432	0·437
Protein N	0·215	0·271	0·308	0·277	0·287	0·424	0·286	0·245	0·238	0·445	0·398	0·292	0·398	0·321	0·228	0·267	0·271	0·217	0·237	0·320	0·264	0·327	0·308
Albumin N	0·064	0·081	0·128	0·277	0·209	0·424	0·247	0·129	0·238	0·445	0·108	0·210	0·152	0·021	0·047	0·240	0·015	0·026	0·237	0·230	0·045	0·327	0·110
Globulin N	0·151	0·191	0·180		0·076		0·039	0·114			0·290	0·081	0·246	0·300	0·181	0·025	0·196	0·191		0·090	0·219		0·198
Non-protein N	0·198	0·143	0·121	0·196	0·135	0·021	0·148	0·237	0·199	0·121	0·101	0·209	0·126	0·100	0·233	0·190	0·180	0·243	0·114	0·116	0·179	0·105	0·129
In milk sample Solids-not-fat %	6·06	6·08	6·59	6·75	6·51	6·56	6·56	6·96	6·88	6·74	7·77	7·65	8·13	7·82	7·82	7·52	7·44	6·81	8·03	8·10	7·71	8·10	8·05
Cl	0·221	0·218	0·190	0·182	0·204	0·201	0·197	0·193	0·176	0·195	0·154	0·160	0·157	0·130	0·136	0·142	0·157	0·189	0·120	0·115	0·108	0·094	0·104

Cow ...	F	E					M						Bulk (*b*)			Average of 38 samples		Ox blood serum
	19. iv. 32*a*	19. iv. 32*a*	3. v. 32*a*	31. v. 32*a*	17. v. 32*a*	14. vi. 32*a*	4. vi. 32*a*	11. vi. 32*a*	8. v. 32*a*	20. iii. 32*a*	21. v. 32*a*	16. v. 32*a*	9. iii. 31*a*	10. iii. 31*a*	4. v. 32*a*			
Dilution %	19·7	10·0	10·0	10·8	4·0	4·1	2·6	1·3	3·4	8·9	2·6	3·1	1·6	2·6	4·1			
%																(s.d.)		
Total N	0·498	0·499	0·514	0·545	0·527	0·517	0·492	0·416	0·482	0·444	0·481	0·503	0·431	0·437	0·473	0·458	0·036	0·440–0·550
Protein N	0·195	0·263	0·443	0·445	0·490	0·063	0·415	0·262	0·032	0·363	0·200	—	—	—	0·080			0·410–0·510
Albumin N	0·163	0·206	0·206	0·244	0·490	0·063	0·415	0·092	0·032	0·363	0·165	—	—	—	0·080			0·290–0·420
Globulin N	0·032	0·055	0·237	0·199				0·161			0·035	—	—	—				0·080–0·150
Non-protein N	0·304	0·236	0·071	0·100	0·038	0·454	0·077	0·154	0·450	0·081	0·281	—	—	—	0·393			0·030–0·040
In milk sample Solids-not-fat %	8·94	8·64	8·97	9·15	8·97	8·78	8·06	8·08	8·02	8·08	8·03	8·14	8·54	8·83	—			
Cl	0·072	0·092	0·091	0·087	0·088	0·102	0·100	0·128	0·124	0·134	0·117	0·119	0·106	0·106	0·073			0·273–0·303

The diluent thus may be taken to conform in composition either with a modified form of blood or lymph serum or a casein- and lactose-free type of milk, and the deficiency in solids-not-fat in the samples may be due to one or more of the following causes: (*a*) a lack of efficiency in manufacturing the required concentration of phospho-protein, (*b*) a lack of efficiency in manufacturing the required concentration of lactose. Reasoning from osmotic relationships, since the chloride content accounts for 75 per cent. of the osmotic pressure (the equivalent of the "cryolac" number) and if the diluent has an abnormally high non-protein nitrogen content, it follows that this nitrogen may to a large extent be in complex combination, *i.e.* as polypeptides with perhaps some as proteose, or generally as complex protein degradation products which in normal milk may have been utilised for building phospho-protein. (This point is being further studied.) The inability to secrete the normal amount of lactose would be counterbalanced by the replacement of the deficit by an isotonic amount of ionised chloride which would explain the high values for chlorides.

Carlson, Greer and Luckhardt (3) have stated that lymph contains more chlorides than blood serum, Binet and Justin-Besançon (2) quoting a value of 0·450 per cent. for whole lymph and 0·365 per cent. for lymph serum. The amount of albumin and globulin nitrogen is also lower in lymph than in blood serum. The higher value for chloride in the diluent together with the lower protein nitrogen leads one to state that there is more agreement in composition with lymph than blood serum. Again, Abderhalden (1) states that certain liquids secreted by endothelial tissue, called "transudates," are closely related to the lymph. Usually these liquids are poorer in protein and carbohydrate and under normal conditions are formed only in small quantities but increase enormously in amount under pathological conditions.

An accompanying condition of milk during and following an attack of mastitis is the secretion of high chloride and a complementary low lactose. This may be explained by the inflamed secretory membranes secreting large amounts of "exudates" rich in chloride. After an attack of mastitis, the membranes might recover from the purulent stage but still remain in a quasi-pathological or catarrhal condition, during which considerable amounts of chloride would be actively secreted among other compounds as a "transudate." The past history of the cows yielding milk of very low solids-not-fat revealed that they had suffered from mastitis (Minett, private information on cows A, B, C and D). It seemed, therefore, possible that a prolonged after-effect of udder inflammation in this manner was demonstrating its presence by the secretion of a diluent of such a composition along with a certain proportion of normal milk and that the amount of this secretion varied in individual animals. The physiological aspects of the problem are being further studied.

SUMMARY.

Casein nitrogen has been shown to account for roughly 76 per cent. of the total nitrogen in over 200 samples of normal milk. Samples low in solids-not-fat have shown a casein nitrogen percentage below this figure, and by assuming a 76 per cent. level of casein nitrogen as standard, it is suggested that milk may be considered as made up of two fractions, (*a*) a true-milk fraction and (*b*) an isotonic diluent. From the distribution of the nitrogen in whole-milk samples it has been possible to calculate on this basis the amount of dilution and the nitrogen distribution in the diluent. Results are given for 38 samples of milk. The chloride content of the diluent has also been approximately arrived at and is approximately isotonic with a hypothetical non-lactose-containing milk.

The similarity in composition of the diluent either to a modified blood serum or lymph serum or a casein- and lactose-free lacteal secretion has been discussed. Its high chloride content favours its similarity either to lymph serum or a "transudate" or "exudate" closely allied in composition to lymph serum from pathological secretory tissue. The possibility of after-effects of attacks of mastitis causing a chronic catarrhal condition of the udder being the cause of the secretion of this diluent is also discussed.

The author is indebted to Mr C. E. Lesser for collecting the samples of milk and for their proximate analyses, and to Mr G. L. Peskett and Capt. W. L. Little for various suggestions.

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66. SOME OBSERVATIONS ON COW'S MILK POOR IN NON-FATTY SOLIDS

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During an investigation (7) in this Institute in which a large number of milk samples from individual cows were found to be poor in non-fatty solids, it was observed by one of us (G. L. P.) that, in most cases, such milks were also poor in fat. This suggested the possibility that the low level of fat and non-fatty solids might be due to a dilution of the normal secretion in the udder by some fluid other than milk.

Earlier studies of the milk of cows suffering from certain inflammatory conditions of the udder point to the conclusion that the milk may be, in such cases, contaminated to some extent with blood constituents. Thus Amberger (1), analysing milk from inflamed udders usually found low levels of fat and lactose, while the chlorine and total nitrogen contents were higher than normal. Seel (11) noted that the total protein content of milk from the affected quarters of cows suffering from mastitis was often high, though the casein content was low, the "albumin" content being unusually high (the term "albumin" probably designates the total content of albumin + globulin). In addition a diminished lactose content was found by Seel to be characteristic of the abnormal milk. The results of Zaribnicky (12) show general agreement with the findings noted above. The significant fact that such milks were often sub-normal in content of casein and lactose while containing more than the usual proportions of albumin + globulin and of chlorides, gives strong support to the theory that in these cases the secretions may justly be considered to consist of normal milk diluted with some body fluid containing constituents characteristic of blood.

In the case of cows yielding milk poor in non-fatty solids it may be, then, that changes have taken place in the cell membranes of some of the milk-secreting cells allowing the passage of a proportion of certain blood constituents to which normally these membranes are relatively impermeable. Such modification of the cells would explain the changes in the composition of milk which have been noted in mastitis. Although in the investigation reported below we are not yet able to state with certainty how many of the cows had been, or were suffering from mastitis, the fact that this herd had been producing milk unusually poor in non-fatty solids provided an opportunity of testing the "dilution theory."

Experimental.

The work was carried out on the blood and milk of cows in a herd of British Friesians, and may be subdivided under two headings.

I. *Detection of blood serum albumin in milk.*

It is generally believed that lactalbumin, though similar to, is not immunologically identical with blood serum albumin, while lactoglobulin and blood serum globulin are probably identical. A specific test for the detection of blood serum albumin in milk was therefore sought, as a positive result in such a test would provide convincing evidence that leakage of blood constituents into the milk had occurred.

On March 9 and 10, 1932, milk samples taken on March 8 from a number of cows in the herd under investigation were tested as follows:

The precipitin reaction was employed, using as antibody the blood sera of rabbits which had been previously immunised with pure serum albumin (bovine). Clear filtrates were prepared from the milks by precipitating the casein according to the method of Moir (9). These were brought to *p*H *circa* 6·0 by addition of dilute ammonia. The presence of antigen (*i.e.* blood serum albumin) in the milk filtrates was sought by the usual technique of precipitin testing, several dilutions of antibody being used. At a very early stage it was found that milk samples from a number of apparently normal cows in the Institute herd in all cases gave a slight positive result in this test, from which the conclusion may be drawn that "normal" milk contains traces of blood serum albumin. The results suggested that these were only minute traces, nevertheless they were sufficient to prevent a clear-cut distinction between positive and negative results, such as would have been observed had there been no blood serum albumin in "normal" milk.

The animals were therefore divided into three groups: positive, doubtful and negative, the milk of those in the last group showing not more than the "normal" very slight reaction with anti-albumin serum (Table I).

Table I. *Tests for presence of blood serum albumin in milk.*

Positive		Doubtful		Negative	
Cow No.	Non-fatty solids in milk %	Cow No.	Non-fatty solids in milk %	Cow No.	Non-fatty solids in milk %
5	8·88	25	8·84	21	9·75
9	8·75	24	8·75	15	8·91
11	8·55	26	8·60	19	8·88
7	8·23	23	8·31	1	8·77
8	8·08	27	8·15	18	8·73
12	7·96			17	8·60
2	7·63			16	8·40
10	7·05			14	8·40
6	6·98			20	8·28
				22	8·18
				13	8·05
Mean (9 cows)	8·01	Mean (5 cows)	8·53	Mean (11 cows)	8·64

From the *mean* figures shown in Table I it may be concluded tentatively that the content of blood serum albumin was increased in the milks poorest in non-fatty solids, whilst not more than the "normal" content of blood albumin was demonstrable in the milks richest in non-fatty solids. Between these extremes the mean figure for non-fatty solids was intermediate. If the *individual* figures are considered, however, it will be seen that the correlation is far from perfect between high non-fatty solids and low serum albumin contents. The clearest positive tests were those of Nos. 2, 5 and 6 and the clearest negative Nos. 1, 21 and 22. The number of samples tested was obviously too small to justify a definite conclusion and, owing to its many imperfections, the test (as described in this paper) has been abandoned, though we consider that these preliminary results indicate a direction of study which merits further exploration.

As stated above, several workers have put forward the theory that increase in the "albumin" content of milk may be due to presence of blood protein. Our results give the first experimental proof of the presence of *blood* serum albumin in this "albumin" fraction and show also that traces of blood serum albumin occur in milk which, according to all standards at present known, must be termed normal.

II. *Comparison of the composition of milk poor in non-fatty solids with that of a mixture prepared from normal milk and blood serum.*

An attempt was made artificially to imitate the milk given by cow No. 2, chosen for its low content of non-fatty solids, by dilution of a sample of milk from cow No. 1 (considered to approximate to the normal for a British Friesian) with such a volume of blood serum from No. 2 that the lactose contents of the artificially modified milk and of milk from No. 2 were identical. A similar experiment involving cow No. 3 (normal) and cow No. 4 (poor in non-fatty solids) was carried out simultaneously. It should be pointed out that the selection of these cows was made on the basis of their previous records of non-fatty solids, but that any such definitions as "normal" must, at present, be considered as purely arbitrary.

On March 13, 1933, samples of the afternoon milk were collected from all four cows. Shortly after milking, blood samples from the same animals were obtained from the jugular vein. After a journey by road of some 36 miles, the milk samples were left overnight in a refrigerator and the blood samples in an incubator at 37°. On the next morning the latter were transferred for an hour to the refrigerator, the sera then poured off from the clots and centrifuged. The lactose content of each of the milk samples was determined as quickly as possible by the method of Fine (5). From these results the dilution factors were calculated for each of the artificial mixtures of blood serum and milk, and the mixtures prepared.

Analyses were performed on each of the four milk samples (1 to 4) and on

the two artificially modified samples (2*a* and 4*a*). Distribution of nitrogen was determined by the method of Moir (9) as slightly modified by Davies (2); chloride by the method of Davies (3); total solids and ash by the usual gravimetric procedure and fat by the Gerber method. Table II records the principal results that have been obtained.

Table II. *Analysis of natural and artificially modified milks.*

The results are expressed in mg. per 100 g. fluid.

	Natural milk of Cow No. 1 "Normal" non-fatty solids 1	Natural milk of Cow No. 2 Poor non-fatty solids 2	Artificial mixture of 93·7 % milk of Cow No. 1 + 6·3 % blood serum of Cow No. 2 2*a*	2*a* Calc. (see foot-notes)	Natural milk of Cow No. 3 "Normal" non-fatty solids 3	Natural milk of Cow No. 4 Poor non-fatty solids 4	Artificial mixture of 80·7 % milk of Cow No. 3 + 19·3 % blood serum of cow No. 4 4*a*	4*a* Calc. (see foot-notes)
Total N	469	450	534	541*	514	516	666	663*
Protein N	436	396	509	—	493	475	639	—
Casein N	310	307	305	290†	412	377	353	332†
Albumin N	52	28	92	—	51	47	269	—
Globulin N	75	61	112	—	30	51	17	—
Non-protein N	33	55	25	—	21	41	27	—
Chloride	116	139	129	—	85	153	132	—
Ash	672	671	665	—	662	613	720	—
Lactose	4560	4270	4300	4270†	5070	4090	4040	4090†
Fat	3800	3670	3470	3560†	4570	4370	3770	3690†
Non-fatty solids	8230	7780	8460	—	8890	7970	8900	—

* Calculated from total nitrogen of serum and of milk used to prepare mixture.

† Calculated from dilution of milk used in preparing mixture.

From the results recorded in Table II it is clear that only in respect of casein-nitrogen concentrations do the nitrogen distributions of the artificially diluted milks 2*a* and 4*a* resemble those of the corresponding natural milks 2 and 4. In other respects the divergences are wide. Hence if the dilution theory outlined at the beginning of this paper has any foundation whatever, the diluting fluid is certainly not blood serum itself. That there is good reason, however, for regarding the milk poor in non-fatty solids as consisting of a mixture of normal milk with a diluting fluid is strikingly illustrated by the table, wherein it is evident that, though the dilution factors necessary for preparing the fluids 2*a* and 4*a* were calculated from lactose concentrations, nevertheless in each case almost identical results would have been obtained had casein-nitrogen concentrations been used instead.

Our experimental findings are confirmed by those of Davies (2, 4) reported in this *Journal*. They show that the composition of the diluting fluid includes, in addition to water, chlorides and non-protein nitrogenous substances in greater concentrations than in blood serum. Though there are reasons for doubting the validity of the albumin and globulin figures (*vide infra*), it appears that, at any rate in the case of one of the milks examined (cow No. 4) the diluting fluid also contained blood proteins (in concentration less than in blood serum)—a result to be expected from the first experimental part of this communication. The composition of the diluting fluid is thus very suggestive of such liquids as oedema fluids or inflammatory exudates. In the former, according to Peters and Van Slyke (10), the protein content is either nil or

much less than, the chloride 10–15 per cent. more than, and the osmotic pressure 20–30 mm. Hg less than that of the blood plasma.

It is interesting to consider briefly the implications of this finding. The reducing substances (particularly sugar) in transudates are negligible in amount in comparison with the salts, so that if dilution by a fluid resembling oedema fluid occurred in the case of abnormal milk, the lactose concentration would decrease and the salt (principally chloride) concentration would increase according to the degree of dilution. A brief survey of figures that have been obtained for normal milk from the typical herd of Shorthorn cows at this Institute (by Golding, Mackintosh and Mattick (6) and by Davies (2)) reveals the fact that the lactose and chloride contents are remarkably constant. If this be the case, then the finding of Mathieu and Ferré (8) (lactose concentration + 11·9 × sodium chloride concentration = constant) is as easily explicable on the basis of a dilution theory such as we have suggested as on the basis of a "law" of isotonicity between blood and milk. Indeed, the existence of Mathieu and Ferré's constant may depend on the fact that the sugar content of bovine blood is relatively negligible.

One other point of interest emerges from Table II. Since blood serum contains no casein, it is evident that the theoretical concentration of casein-nitrogen in each of the mixtures 2*a* and 4*a* can be easily calculated. The calculated values are lower by 5–6 per cent. than those actually found by use of the analytical procedure of Moir. This discrepancy could be accounted for quantitatively on the assumption that the casein-nitrogen figures as determined by Moir's method are slightly too high (possibly owing to adsorption of globulin by the precipitate of casein), particularly in the case of milks rich in globulin. Inspection of Table II also shows that the globulin figures (and hence the albumin which is calculated by difference) must be subject to some additional error in the case of liquid 4*a*. In fact, analysis of fluid 4*a* revealed the presence of less globulin than would be expected had the diluting fluid been water instead of serum. Some doubts regarding the accuracy of figures for the nitrogen distribution of milk, as determined by Moir's method, arise from the discrepancies which we have noted above, but before definite conclusions can be reached further work will obviously be necessary.

In conclusion, it is our opinion that the poor quality of many of the milks investigated may be due to some change in the cells of the mammary gland, allowing the passage of a transudate resembling oedema fluid into the milk. Such a change appears to us to be pathological rather than physiological and might be expected if the mammary gland were damaged, either as the result of previous disease or owing to an existing condition of mild, chronic mastitis. We must emphasise that we do not suggest that our conclusions can be applied to the case of all milks which are poor in non-fatty solids, but that in such cases they should be borne in mind as a possible explanation. Moreover, the existence of pathologically "diluted" milks containing a normal or even a high content of non-fatty solids is possible, since the diluting fluid may be richer or poorer in these substances than normal milk.

SUMMARY.

1. Traces of blood serum albumin have been demonstrated in normal milk.

2. The content of blood serum albumin is increased in many of the milks which are poor in non-fatty solids and there is evidence of some correlation between low non-fatty solids and high blood serum albumin content of the milk.

3. The experimental data support a conclusion that deficiency of non-fatty solids in milk in some cases may be due to dilution of a normal secretion in the udder by a fluid of the nature of oedema fluid, *i.e.* to a pathological rather than a physiological condition.

4. Some evidence, which obviously needs confirmation, has been adduced indicating that the casein-nitrogen content of milk as determined by Moir's technique may be slightly too high. The accuracy of the globulin figures seems also to be open to doubt.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE[1]

SECTION B. BACTERIOLOGY AND MYCOLOGY APPLIED TO DAIRYING.

I. Technique of milk examination.
  (*a*) The plate count.
  (*b*) The Burri smear method.
  (*c*) Breed direct microscopic count.
  (*d*) Methylene-blue reductase test.

II. Saprophytic flora of milk and milk products.
  (*a*) Coliform organisms.
  (*b*) Organisms and mechanisms producing faults in milk and milk products.
  (*c*) Lactic acid bacteria.

III. Pasteurisation of milk.

IV. Butter.

V. Cheese.

I. TECHNIQUE OF MILK EXAMINATION.

With the increase in the willingness of wholesalers and retailers to pay an enhanced price for milk of good hygienic quality, and with a general quickening of interest in the whole subject of milk hygiene, an increasing sense of uneasiness, particularly in England and America, as to the accuracy of the methods used is evident from a survey of the literature which has appeared within the last two years.

(*a*) The plate count.

This attitude is nowhere more pronounced than in the matter of the plate count, which is the only method at present available for estimating small numbers of bacteria in milk. Those engaged in the control of milk supplies have for long appreciated the necessity for the standardisation of technique, and *Standard Methods of Milk Analysis*, of the American Public Health Association and the *Guide to the Conduct of Clean Milk Competitions*, of the Ministry of Agriculture bear witness to this appreciation. In these publications details of media preparation and technique have been laid down and, except for the inadequacy of the agar medium (a point which is considered later), there is no reason why results more concordant than are secured at present, and representing more nearly the actual numbers of bacteria in milk, should not be obtained, if all workers recognise the possibilities of error and take

[1] This review has been compiled from the literature which has appeared within the period December, 1930 to December, 1932, but a few earlier papers have been referred to when necessary.

In the section devoted to pasteurisation machinery has been dealt with, but only in so far as its efficiency has been assessed by bacteriological methods. The study of pathogenic organisms as such, does not come within the scope of this review.

the steps necessary to control or eliminate them. The failure of workers to appreciate the absolute necessity for attention to detail is well brought out by Schacht(1), who draws attention to a number of points which are ignored in many laboratories. For example, it is pointed out that sampling, a very important factor, is usually not under the control of the bacteriologist and is often inadequately carried out. Devices which aim at a thoroughly representative sample of a given bulk of milk have been described by Keith and Walters(2), and Barnes and Bousfield(3). The former emphasise the fact that the distribution of bacteria in milk in which the cream has been allowed to rise is very irregular. This is probably due to the fact that the fat globules enmesh many of the bacteria and take them to the surface. Schacht(1) further mentions the lack of care so often encountered in the preparation of dilution blanks. It is clear that the accuracy of a count must depend largely on that of the dilutions used and their accurate measurement is essential. The only method of securing this is by filling out after sterilisation, to avoid loss by evaporation.

The method of calibration and use of pipettes affect the accuracy of a plate count, and Schacht(1) observes that some workers alternate draining and blowing when using pipettes calibrated to deliver. The difficulty of inaccurate deliveries has been to some extent surmounted by the use of pipettes calibrated to *contain* the required volume, since such pipettes can also be used for mixing milk and diluent in test-tubes. The fluid adhering to the outside of the pipette is removed by plunging through a sterile filter-paper fixed over a beaker or jar, and pierced in many places with the tip of a scalpel. The piercing of the paper avoids the withdrawal of fluid from the lumen of the pipette as it passes through(4). The method, whilst not ideal, is practical and at least uniform.

Owing to the cost of the plate count it has often been suggested that the number of plates made from each sample might be reduced. Schacht(1) draws attention to some of the objections to this practice, such as overcrowding, "spreaders" and lack of any check. There are, however, even more substantial reasons why such practice should be condemned. Malcolm(5), using the technique laid down by the Ministry of Health, has worked out the correlations between the means of the number of colonies, of two, five, ten, and that of twenty plates of the same dilution of the same sample of milk. He suggests that five plates be made from the 1/100 dilution of milk containing from 50,000 to 60,000 organisms per 1 ml. If the counts are in the neighbourhood of 100,000 per 1 ml., Malcolm suggests the use of at least five plates of the 1/1000 dilution. It would seem that such a method could only be applied to a milk supply with which the worker was well acquainted and would in general practice be costly, but the paper directs attention to the folly of endeavouring to restrict the number of plates. It is nevertheless certain that there would be overcrowding in plates of the usual size, when 500–600 colonies were present, and the counting error would be considerable.

The actual counting of the colonies on plates is a factor of great importance in the accuracy of the final result. Schacht(1) has drawn attention to this fact and recommends the use of a lens and a suitable method of illumination. It had been found that extreme variation occurred when the same plate was counted by different workers, and to find the extent of the variation in the experiments briefly reported in the paper, a number of persons using various devices (presumably for illumination) counted a number of plates. The results showed that the same person counting one plate may reasonably be expected to show a variation of not more than 5 per cent. When different persons count the same plate the figure becomes 10 per cent. Illumination of the plates with artificial light invariably resulted in higher counts than with daylight. Lenses are regarded as essential, but the mode of their use is at present unspecified. Hiscox and Mattick(6) secured evidence, in testing a newly-

devised counting chamber, that the great variability noted by Schacht when several persons count the same plates in daylight was reduced to reasonable proportions when all those engaged in counting used identical counting chambers, of the type under test, and lenses and tally counters all of the same specification. They found, however, that the arithmetical spread tended to increase as the number of colonies on the plate increased. It is clear, therefore, that the actual counting of the colonies on the plates may introduce such serious errors as to merit very close consideration. Mechanical devices for recording the colonies counted should go far towards eliminating errors in actual counting. An example of such a device is given by Robinson(7). Spreading colonies appear to cause great difficulty in many laboratories and numerous suggestions for their control have been made. Schacht(1) suggests that the various types of "spreaders" result from delayed hardening of the agar, or from pouring agar which is too cold, and recommends a temperature of 44–46° C. for pouring. Boerner and Robinson(8) claim to have shown that the incidence of "spreaders" is not due to the samples, the incubators, contaminated agar, the water or the air, but attribute the trouble to the volume of the agar used. They claim that 15 or 20 ml. of agar is too much and that a volume of 10 ml. gives satisfactory results. It must be stated that this is by no means true in all laboratories, and Broadhurst and Krug(9) using 10 ml. quantities noted the occurrence of "spreaders." These authors claim that the percentage of agar in the medium is a controlling factor. They used 3 per cent. agar and found it satisfactory. It seems that the problem of "spreaders" is not a simple one and that the solution in all cases is not yet known.

It is well known that the temperature of incubation generally used in milk work (37° C.) is purely arbitrary, and that the plate count is influenced by the temperature. It is not generally recognised that seriously uneven temperatures, due to a variety of causes such as failure to circulate the air and overcrowding with plates, may occur in incubators of ordinary types (Mickle(10)). Breed and Pederson(11) have shown that there may be enormous variations in temperature (from 46 to 33° C.) in different parts of the same incubator with electrically heated plates. Such variations caused great differences in plate counts, *e.g.* when the average number in a well-controlled incubator was 38, the highest in the incubator under test was 62 and the lowest 0. In water-heated incubators it was found that the temperature never exceeded 37° C., but plates heated very slowly and even in 48 hours some did not reach 37° C. Anhydric incubators heated more quickly, but the plates finally became too hot. The authors suggest that large incubators fitted with fans, which cannot be overcrowded, are best. Some shelves in such an incubator may be expected to keep a constant temperature. The writers' own experience confirms this suggestion, since good results have been secured with a gas-heated incubator room lined with asbestos sheets and lagged with glass-wool. The air is admitted at floor-level and circulated by means of a fan in the roof. Even so it is only on some shelves that the desired temperature is maintained. Jennison(12) has described an incubator of simple construction which is claimed to maintain a uniform temperature within $\pm$ 0·15° C. up to 60° C. In this case also arrangements are made for admitting and circulating air. It is evident that the problem of efficient incubator construction still requires serious consideration.

The factors influencing the accuracy of the plate count which have so far been considered are wholly or largely within the control of the careful worker. The fundamental question of the nutrient medium to be used is, however, one of some difficulty. No truly synthetic media suitable for the requirements of the miscellaneous flora of milk have so far been found, since they lack some essential but unknown factors. The ideal medium would be a truly synthetic one reproducible in all laboratories, but failing this, it is clear that, for the moment, uniformity can only be

secured by distributing all ingredients of variable composition from a common bulk(4).

It is quite clear that for any but the very cleanest milk, the present media used by the Ministry of Health in England(13) and by the A.P.H.A. in America(14) are inadequate. This is particularly so for pasteurised milk(15). McIntosh and Whitby(16), working with pasteurised milk, noted the inadequacy of standard agar and obtained largely increased counts on a medium incorporating meat extract, peptone and lactose. Hiscox *et al.*(17), using standard agar to which sterile separated milk had been added at the time of plating, also found enormous increases in the plate counts of some samples of raw milk and many samples of pasteurised milk over those obtained on standard agar alone. They showed that neither casein-peptone (digest), yeast extract, lactose, dextrose nor galactose could satisfactorily replace milk as an addition to standard agar. They also found that lactose, whilst often causing increases, sometimes caused significant decreases in comparison with the count on standard agar. They suggested that some of the thermophilic bacteria of pasteurised milk were unfavourably influenced by this sugar. The standard agar, reinforced by milk, gave colonies which were more dense, developed more quickly and were easier to count. This probably accounted for the fact that increased incubation times resulted in a smaller percentage increase of countable colonies, *i.e.* although 48 hours at 37° C. is an inadequate time, it is less so in the case of standard agar and milk than in that of standard agar.

Demont and Dorner(18) compared the value of trypsinised blood, trypsinised milk and diluted whey freed from albumin, as nutrients with agar as a base. They were all of about equal value and gave increased counts over those on meat infusion agar. Witte's peptone was successfully replaced by a cheaper one used in conjunction with whey. Devereux(19) noted the failure of many milk bacteria to grow on plain nutrient agar and recommended a medium in which yeast extract, peptonised milk and dextrose were incorporated.

The fact that a number of media for use in milk examination have been devised and tested during the last two years points to the dissatisfaction of workers with the media in use at present. It appears, however, to have escaped attention that the first requirements for a standard medium are simplicity, cheapness and, above all, reproducibility. Since nearly all the media prepared contain substances of indefinite and variable composition, the essential of reproducibility cannot be fulfilled.

Even with media containing milk, which seem to approach the ideal most nearly, it can be argued that milk itself is variable in composition. Nevertheless if milk which is destined for use for media reinforcement is drawn from a large bulk it is unlikely that serious differences will be encountered.

(*b*) The Burri smear method.

The simplicity and economy of this method have caused considerable interest since its description by Burri(20).

One of the first questions which naturally arose was that of its accuracy in comparison with the plate count. Dorner and Demont(21), after a detailed description of Burri's technique, discuss the calibration of the platinum loops, and compare the results and variations of determinations made by Burri's and by a plating method. It appears that the use of loops with milk or milk dilutions has certain fundamental objections which do not seem to have been altogether overcome. The authors recommend incubation of the inoculated tubes at 30° C. for 48 hours, and note that longer incubation yields results from 10 to 20 per cent. higher, and that overcrowding and "confluence" of colonies may constitute difficulties. Since it was

noted that streak cultures of milk of low bacterial count often gave higher counts than the plate method, it was believed that disintegration of bacterial clumps was caused by the process of streaking. A number of samples of milk were therefore homogenised, plated and streaked by Burri's method and the results compared with those of the same milk not homogenised. Assuming the feasibility of cleaning a homogeniser thoroughly it is of interest to note that the plate count, even of milk of very low initial bacterial content, is materially increased by homogenisation, a fact which emphasises the undesirability of using the term "bacterial count." Clearly, "colony count" is the only safe method of expression.

In a second paper Dorner and Demont(22) have made comparisons on a statistical basis of the results of the examinations of parallel determinations by the plate method and by Burri's method. They claim to have established that the degree of dispersion of the results of parallel determinations is of the same order by both methods. Cunningham(23), however, has pointed out, that many of the investigations discussed above were made on broth cultures of *Str. lactis* and doubts the applicability of the results to milk because the errors may not be comparable. Further, the use of unmeasured quantities of agar, the division of the plating work amongst a large number of workers, the certain errors in enumeration of colonies on the plates from person to person and the apparent lack of uniform illumination probably exaggerate the errors of the plating technique and cause them to approach nearer to those of the Burri method. Cunningham also found in examining thirty samples of milk, making ten plates and ten Burri smears in each case, that the average percentage error of the plate method was 4·34, whilst that of Burri's method was 9·22, and he concludes that the error is so large as to preclude the use of this method for making accurate determinations of bacterial numbers in milk samples.

It may be observed that Burri smears are really milk agar cultures, and that if a more adequate medium (Hiscox *et al.*(17)) were used for plating, still further differences in the two methods might be observed.

As a method for the control of starters (Knudsen(24)), and for the detection of gross contamination in production and distribution, Burri's method is excellent, but the writers believe that it is unlikely in its present form to replace the plate count for accurate enumerations.

(*c*) Breed direct microscopic count.

The direct microscopic method of milk examination has been generally accepted as a very useful one under certain conditions. It has the advantage of giving direct information as to the past history of and types of organisms present in milk and is of great value in revealing the presence of thermophiles in pasteurised milk, provided examinations are made before as well as after heating. It cannot be used for determining actual numbers of living bacteria in heated milk as it does not distinguish between living and dead organisms. In practice it is also inapplicable to really low-count milk because of the impossibility of examining sufficient fields and the overpowering influence of the multiplication factors which are used (Robertson *et al.*(25)). Nevertheless in separating good milk from bad it is of great value.

It must be recognised, however, that the accuracy of the method may in some cases be vitiated by the failure to stain of certain organisms commonly present in milk. Whitehead(26) has shown that mature or old Gram-negative organisms of coliform type added to milk from solid media (in practice, milk deposits) do not stain and cannot be detected. This author suggests that direct microscopic examination may in certain cases give a false picture of the numbers and the distribution of the types present, since the older Gram-negative bacteria will not stain.

A new differential stain which, like Newman's, incorporates tetrachlorethane, has been described by Broadhurst and Long(27). The stain, which colours bacteria and white cells red against a grey-green background, is stated to keep well and to give results which compare favourably with those of methylene blue.

Pien and Baisse(28), discussing the technique of direct enumeration, suggest the use of a mixture of five parts of milk in one part of 50 per cent. $CaCl_2$ solution for making films. They claim that much more even films, easy to examine microscopically, are secured by this method.

(*d*) Methylene-blue reductase test.

Of all the methods which have been advocated for the control of the quality of milk none is more attractive, on the grounds of simplicity and economy, than the methylene-blue test. In its proper sphere it has been of the greatest value, but many workers have endeavoured to interpret results in terms of the plate count since first the very broad grades of count, to which reduction times generally corresponded, were suggested. There is no doubt, however, that this method of interpretation is doomed to failure, as the two tests measure different things. The plate count is, within its limitations, a measure of absolute numbers, whereas the reduction of methylene blue is a measure of the activity of the organisms. Recent work on oxidation-reduction potential by a number of authors has firmly established the decolorisation of methylene-blue by bacteria in milk as an oxidation-reduction phenomenon, and it has been shown that the blue compound is reduced at different rates by the different organisms (or their activated metabolites) which occur in milk. Amongst the most rapid reducers are *Str. lactis* and *Bact. coli* (Frazier and Whittier(29)). The latter is at its optimum at the usual temperature of the test. Organisms like *B. subtilis* are not such active reducers, and it appears that the ease with which the metabolites are activated is more closely connected with methylene-blue reduction than with mere numbers of bacteria. It must therefore be clearly understood that the reduction time of methylene-blue is influenced by a number of factors—temperature of incubation, type of organism (the nature of its metabolites) and the rate of growth in milk. Attempts to correlate plate counts with reduction times at 37–40° C. are therefore certain, in many cases, to fail.

Numerous modifications of the original methylene-blue test have been suggested, chiefly to make it more applicable to the examination of high-grade milk, to which it is at present unsuited (Hiscox and Starling(30)). Johns(32) has shown that the variability between replicates is large in low-count samples, and places the upper limit of accuracy at 10 hours reduction time. Even this does not cover the range of high-grade milk. Johns suggests that preliminary incubation at a temperature lower than 37·5° C. would render the test more applicable to all grades of milk. He points out, quite pertinently, that the organisms which are of the greatest importance are those which grow at the temperature of domestic storage, and for this reason suggested 12·8° C. (55° F.) as a suitable temperature for preliminary incubation, which was arranged with an eye to the convenience of the analyst—an important practical point. Johns took advantage of the observations of Thornton and Hastings(31) that shaking, to avoid the sweeping of bacteria out of the system by rising fat globules, shortens the reduction time, and shook those tubes which were unreduced at the end of 6 hours. Methylene blue was added at the end of the preliminary incubation period of 18 hours, and the tubes held thereafter at blood heat. The keeping quality found by actual testing at frequent intervals was compared with the ordinary methylene-blue test, and with that as modified by Johns. An examination of the scatter diagrams shows that the degree of scatter is less when the modified test is

used. There is thus a better correlation with the keeping quality test; but the degree of correlation is still less than is desirable. Incidentally Johns has pointed out that the acidity test is an unreliable guide to the quality of milk and that in really clean milk the plate count correlates more closely than the standard methylene-blue test with keeping quality.

Pursuing further the suggestions of Johns, Hiscox *et al.*(17) have, in a preliminary note, proposed the use of the methylene-blue test at 15·5° C., without preliminary incubation. Their results, though incomplete, indicate that there is good correlation between reduction time and keeping quality as determined by taste. The rate of change is convenient, and milk of poor quality causes comparatively quick reduction. Erkkilä(33) has made the interesting observation that the addition of ethyl alcohol in the reductase test results in some cases in a shortening of the reduction time. Although the author does not discuss it, it is possible that this is due to the dispersion of the fat in solution in alcohol, resulting in a better distribution of bacteria such as occurs when the samples are shaken.

The use of the reductase test at 63° C. for the detection of thermophilic bacteria both in raw and pasteurised milk continues to attract attention (Alfonsus and Correll(34)) and its value is being increasingly recognised.

Finally, Whitehead(35) has made the important observation that light catalyses a mechanism involving unsaturated fatty acids responsible for the reduction of methylene blue in the absence of bacteria, and has emphasised the necessity of performing the methylene-blue test in the dark.

In general it appears, in spite of improvements, that as a measure of bacterial numbers, the methylene-blue reductase test cannot yet be advocated as a basis for payment according to hygienic quality, as the distinctions between really clean milk and other milk are not sufficiently fine. It is, however, unreasonable to expect agreement between bacterial numbers and reduction times, but until the question of activity has been resolved for a large number of bacteria commonly found in milk, it is possible that attempts will continue to be made. Meanwhile there appears to be no good reason for continuing to use the more expensive plate-count method for milk containing more than say 500,000 bacteria per 1 ml., since it has been shown that the methylene-blue test in these ranges is probably at least as accurate as the plate count as at present used (Robertson *et al.*(36)).

II. SAPROPHYTIC FLORA OF MILK AND MILK PRODUCTS.

(*a*) Coliform organisms.

The presence of large numbers of coliform organisms in milk is generally taken to indicate improper methods of handling and the need for reorganisation of methods and adequate sterilisation of equipment in order to reduce the numbers in milk is being increasingly realised. The influence of coliform organisms on the keeping quality of milk has been studied by Hoy and Newland(37) who correlated the presumptive coliform content of pasteurised milk with the keeping quality, irrespective of the total count. It has been shown by these workers that milk of low bacterial count may have a very poor keeping quality, and that this lowering of keeping quality is almost invariably connected with the presence of a high proportion of coliform organisms. Chalmers(38) investigated a typical case and traced the low keeping quality of milk from a certified herd to the presence of organisms of the *Bact. aerogenes* type, probably of faecal origin. These were shown to gain access to the milk by infection of the surface of the udder and the teat canals between milkings.

The results of Whitehead and Cox(39) are interesting in this connection, for they have been able to show that coliform organisms grown in milk for 3–16 hours produce substances which can partially inhibit the subsequent formation of acid by lactic acid streptococci. The nature of the inhibitory substances is not clear, but they appear to be able to withstand a temperature of 75° C. for 1 hour without serious modification of their action. The antagonism which may exist between the different members of a milk flora has as yet received little attention, and this contribution opens the way for further studies which may prove of value in assessing the quality of milk.

As regards coliform organisms in water supplies Burke-Gaffney(40) has given a comprehensive review of the subject, and reports fully on the present position concerning the use of coliform tests in assessing the sanitary quality of water in tropical and temperate climates.

Several methods, or modifications of existing methods of estimating the coliform content of milk, have been described. Ruhmekorf(41), in investigating various types of milk in Leipzig, obtained good results with a modification of Klimmer, Haupt and Borcher's trypaflavin method. As in the original method, lactose trypaflavin broth (trypaflavin concentration 1/100,000) was used for demonstrating the production of gas, and lactose brom-thymol blue trypaflavin agar for plating, but in the latter case the concentration of trypaflavin was increased from 1/20,000 to 1/10,000 in order to cut out micrococci. The *coli-aerogenes* colonies, it is stated, could be clearly distinguished from those of other lactose fermenters. Klang(42), in studying gas production by coliform organisms in gentian-violet bile salt lactose peptone solution, was able to show that the medium did not entirely inhibit the growth of other milk organisms, but so modified their development that they did not interfere with the gas production by the coliform organisms. The reaction was measured by the evolution of gas in Einhorn flasks. In examining the dilution factor, he concluded that in this medium, with incubation for 24 hours at 37° C., a minimum inoculation of 60–70 coliform organisms was necessary to produce a definite gas reaction, but that if incubation was continued for 48 hours at the same temperature, a single actively growing organism was sufficient.

Minkewitsch(43) examined the coliform flora of a number of samples of commercial milk using various media, and reported the best results with the medium of Kessler and Swenarton and its modification, the bouillon milk gentian-violet medium. A neutral red-broth medium has been described by Vassileff(44) which may be used for the routine testing of milk. Saline dilutions of 1/50 or 1/100 are used for pasteurised and of 1/1000 or 1/2000 for raw milk. The prime object of diluting is stated to be to reduce as far as possible the amount of those substances present in the milk which may interfere with the reaction. The modifying influence of such substances and the possibility of their occurrence in some samples of milk and not in others, lays the test open to criticism. The dilutions described are, moreover, so high as to make the test useless for revealing the smaller numbers of coliform organisms considered significant in England and other countries. Tonney and Noble(45) have shown that their ferrocyanide citrate agar method may be applied to the examination of milk.

Efforts are continually being made to evolve rapid methods of differentiation within this group, but the results do not seem to do much but add to the confusion which already exists. Increased knowledge of variation, atypical forms and allied phenomena, is shedding new light on bacterial groupings, and in dealing with closely related forms such as the members of the *coli-aerogenes* group, the possibility of evolving any rapid and accurate differential test becomes steadily more remote. Much information of interest has, however, been recorded. Ruchhoft, Kallas, Chinn

and Coulter(46, 47) obtained good results with Levine's eosin methylene-blue medium and Skinner and Murray's crystal-violet modification. It seems that neither was ideal, since difficulty continually arose due to the incidence of mixed colonies and the masking effect of organisms not belonging to the *coli-aerogenes* group. These authors have very wisely suggested that cultures should be purified before any attempt is made at differentiation. In the later paper(47) they showed that preliminary enrichment in lactose broth leads to anomalous results and that it is better, where exact differential results are required, to use cultures direct from plates and to test them without further treatment by the indol, methyl red, V.P. and Koser's citrate methods. Burke-Gaffney(40) found a similar anomaly in examining the *coli-aerogenes* types from water. He observed that preliminary enrichment encouraged the overgrowth of *Bact. aerogenes* and lactose-fermenters not belonging to the *Bact. coli* group, and that Eijkman's method of incubation at 46° C. failed to eliminate these fallacies. It appears that the picture obtained after treatment in bile-salt lactose is not a true indication of the state of affairs in the original milk or water, and caution is required in interpreting the results. The predominance of one or other type during enrichment is due, according to Ruchhoft and his co-workers, to such factors as initial inoculation, lag phase, rate of growth and the effect of products of metabolism. In order to get a true picture of the partition of *coli-aerogenes* types, therefore, plating would seem to be the only solution.

Burke-Gaffney(40) has devised a glucose phosphate peptone agar medium containing brom-thymol blue. With the reaction adjusted to *p*H 6·8–7·0 known cultures of *Bact. coli* and *Bact. aerogenes* can, it is stated, be clearly distinguished from one another. Two hundred cultures from various sources were tested, and it was finally established that *Bact. coli* and intermediate organisms gave orange-coloured colonies, whilst *Bact. aerogenes* and atypical forms gave green. The method gave satisfactory results in the differential plating of water samples. Levine *et al.*(48) studied 401 *coli-aerogenes* cultures isolated from eggs and submitted them to the following tests: citric acid, V.P., M.R., H_2S, indole and fermentation of glycerol, aesculin, salicin, starch and pectin. By this means they were able to divide them into four quite well-defined groups: (1) *Bact. coli*, (2) *Bact. aerogenes*, (3) *Bact. cloacae*, and an unidentified group producing H_2S. Tonney and Noble(49) have described an improved, but still complicated, ferrocyanide citrate medium, on which they claim that it is possible to distinguish *Bact. coli* colonies from those of *Bact. aerogenes*. They state further that an important point in favour of the method is that the results are not interfered with by other common lactose-fermenters such as *Cl. welchii* and *B. asterosporus*. Ruchhoft *et al.*(46) were able to confirm the differentiation, but they emphasised the fact that any deviation from the method described for preparing the medium or the plates would result in failure. It is clear, therefore, that although the ferrocyanide medium has great possibilities in the hands of experienced workers, it presents too great difficulties for use as a routine method. Edmunds(50) has described a medium consisting of Koser's citrate solution to which agar and brilliant green are added. In plating mixed cultures on this medium only colonies giving the reactions of *Bact. aerogenes* developed. *Bact. coli* appeared to be entirely inhibited. The use of dyes requires care, as is shown by Perry's(51) work with Haven's brilliant green bile medium. The selective bacteriostatic properties, in this instance, have been shown to be adversely affected by the addition of peptone or meat extract and by variation in *p*H. Also, the use of crystal violet in presumptive tests for water pollution has been found by Stark and England(52) to give rise to anomalous results. In Salle's medium, 32 out of 95 faecal cultures were inhibited, although all 95 cultures gave positive results when the dye was omitted. Ruhmekorf(41), using a modification of Klimmer, Haupt and Borcher's trypaflavin method, found a certain differentiation

between *Bact. coli* and *Bact. aerogenes* colonies, in that *Bact. coli* colonies were frequently reddish and transparent, whereas *Bact. aerogenes* were larger, slimy and opaque but this difference does not appear sufficiently clear to be of value in differentiating the two species. Another medium, a $CuSO_4$ agar, has been described by Cascelli(53). The *coli* colonies which develop are said to be characteristic and of a yellow or brownish colour, whilst closely related types, notably *Bact. aerogenes* and members of the *coli-typhoid-dysenteriae* group, are inhibited. Two rapid methods of distinguishing between *Bact. coli* and *Bact. aerogenes* have been described by Lindsey and Meckler(54), the first a simplification of Werkman's ferric chloride modification of the V.P. test, in which the incubation period is reduced from 4 days to 24 hours and glucose broth substituted for the original complex medium; the second, the reduction of methylene blue by 24 hours old cultures grown in lactose broth. The latter method showed agreement with the V.P. test in 44 cases out of 45. Using neutral red in place of methylene blue, Wilson(55) has recently obtained results which seem to confirm those of Lindsey and Meckler. This worker has investigated two differential media containing indigo sulphate and arsenious oxide respectively. Both *Bact. coli* and *Bact. aerogenes* produced bleaching of indigo, but the bleaching brought about by *Bact. aerogenes* was more intense. As regards the use of arsenious oxide, it is recorded that 1 ml. of a 1 per cent. solution added to nutrient agar gave a medium on which all strains of *Bact. coli communis* grew abundantly, whilst the growth of many, but not all, strains of *Bact. aerogenes* was inhibited. A difference in the ability of *Bact. coli* and *Bact. aerogenes* types to ferment polyhydric alcohols has been reported by Poe and Field(56) since *aerogenes* types were observed to produce acid from inositol but *coli* types failed to do so. Measurable amounts of gas were produced by certain members of the *aerogenes* groups, but visible gas could never be detected in *coli* cultures. Koser and Saunders(57) have noted a difference in the fermentation of α-methyl-*d*-glucose by the two groups, *Bact. coli* of faecal origin failing entirely to ferment this substance.

Barthel(58) has made the interesting observation that *Bact. aerogenes* produces appreciable quantities of iodine-fixing substances in milk, whilst *Bact. coli* does not do so. These compounds could, he found, be estimated by treating the milk distillate with iodine and NaOH and titrating with thiosulphate. The amount of iodine fixed was expressed in terms of ml. $N/10$ iodine. The value for *Bact. coli* was found to be little above that of the controls (generally below 1), whilst that of *Bact. aerogenes* might be as high as 39. These substances are most probably bound up with the formation of acetyl-methyl-carbinol. Improved methods of testing for acetyl-methyl-carbinol have been advanced by O'Meara(59) and Leifson(60). In the first of these, the culture is tested by adding a small quantity of creatin together with 5 ml. conc. NaOH. A positive reaction is denoted by a red colour which develops in 2 min. In the second an alkaline solution of $CuSO_4$ is added to the culture in equal amount. The appearance of a bright red colour in 10–20 min. indicates a positive reaction. Both these methods have the advantage of being rapid and delicate.

Important variations have been recorded in the ability of certain strains of coliform organisms to ferment lactose. Knoth(61) investigated 28 non-lactose-fermenting strains from meat and intestinal contents and classified them according to their indole reaction and their growth in litmus milk, saccharose and synthetic media. Similar non-lactose-fermenting forms were isolated by Kennedy, Cummings and Morrow(62) from stools and urine samples, whilst Jones, Orcutt and Little(63) obtained atypical slow lactose fermenters from the faeces of cows suffering from intestinal disorders. All these workers seem to be unanimous in assigning such forms to a position intermediate between the true lactose-fermenting coliform types and the non-lactose-fermenting paratyphoids. In this connection Grimes and Hennerty(64)

have made interesting observations on *aerogenes* types isolated from butter. It was found that if these cultures were incubated in lactose broth or litmus milk at 37° C. no fermentation of the lactose took place. If, however, the cultures were incubated at 21° C. typical fermentation of the lactose occurred. These findings have recently been confirmed by Hiscox(65) with a type of *aerogenes* isolated from milk which on manufacture gave gassy Stilton cheese.

(*b*) Organisms and mechanisms producing faults in milk and milk products.

There are several important contributions to record in connection with the oily or tallowy taint of milk. Diernhofer(66), Kende(67), Kertész(68) and Csiszár(69) have approached the problem by somewhat different routes and have confirmed the observation of many earlier workers that the taint is not due to bacterial action, but to the oxidation of certain constituents of the milk fat. The work of Kertész(68) and Richter(70) has shown that certain deficiencies in the ration fed to the cows may predispose milk to the development of taints. It is possible that the variations in susceptibility to oiliness noted in different samples of milk is closely associated with this factor. On the other hand, Csiszár(69) is of the opinion that the feeding has no influence, but rather that the susceptibility is a function of the metabolism of the cow. Widespread interest has been aroused in the problem of oiliness in milk, and the concensus of opinion appears to be that there is very little evidence for its bacterial origin, and that the development of the taint is bound up with chemical and physiological factors. Richter(71) has investigated the action of buffering in connection with milk taints and has described a fat-splitting organism which produced the "lehmig-adstringierend" taint. This organism was not inhibited by the addition of 10 per cent. of starter. The $CaO:P_2O_5$ quotient in tainted milk was found to be 0·93 as compared with 0·99 in normal milk, and therefore allowed of the growth of undesirable non-acid-forming organisms. It was shown that the lactic acid bacteria could be induced to grow if the deficiency were repaired by adding Na_3PO_4 or $CaCl_2$. The ratio could also be improved by feeding chalk and $CaCl_2$ to the cows.

Stark and Foter(72) have examined 500 cultures of organisms causing ropiness in milk. These could be roughly divided into two main groups, Gram-negative bacilli and Gram-positive staphylococci. Pasteurisation was effective in killing these organisms in artificially grown cultures, but the authors state that under natural conditions the resistance is probably much higher. Hammer and Hussong(73) isolated *Bact. viscosum* from various dairy products and found that all strains failed to withstand 61·1° C. for 3 min. Cultures of *Bact. aerogenes* on the other hand showed great variation in heat resistance, old cultures frequently withstanding 62·8° C. for 10 and 20 min. Ropiness, it is concluded, may therefore be due to contamination either before or after the heating process. Stark and Stark(74) have observed that cultures of *Bact. aerogenes* can be dissociated under the influence of small quantities of bacteriophage into a slimy type which produces ropiness in sterile milk. The appropriate bacteriophage has been demonstrated in lettuce, carrot and other plant tissues. It is suggested that bacteriophage may act as a precursive agent in the development of organisms causing ropy milk.

Henneberg(75) has studied in some detail the bitter flavours occurring in milk, butter and cheese, and has listed the organisms which cause these flavours. Chief amongst them were *Bact. aerogenes, fluorescens, cloacae* and *alcaligenes* types, though *Actinomyces* and certain types of *Corynebacterium* might also be responsible. Other organisms, *e.g.* aerobic spore-formers, lactose-fermenting yeasts and certain moulds, although not normally possessing the property, could assume it and produce typical bitter flavours. Bitterness in cheese, it is stated, may be brought about by degradation

of the proteins or by the formation of specific bitter substances by micrococci and corynebacteria. Csiszár(76) has reported a bitter nauseous flavour of milk in which *Ps. fluorescens* was responsible for the bitterness, and *Micrococcus pituitoparus* and *Bact. aerogenes* for the nauseous flavour. The origin of these organisms was thought to be the water used for washing the plant and the milk bottles. Bitterness in a city milk supply has been traced by Hood and White(77) to an atypical *Bact. coli.*

Hiscox and Christian(78) have examined a coconut or carbolic taint of commercial sterilised milk. The causative organism has been shown to be an atypical aerobic spore-former which requires specialised conditions for spore formation. The taint could be reproduced by inoculating tainted milk or cultures of the organism into milk, but unless sporing cultures received a heat treatment the spore form was rapidly lost and replaced by a stable asporogenous form. An aroma resembling that of amyl alcohol produced by the growth of certain white and orange micrococci in milk has been reported by Cunningham(79). The trouble occurred only during the winter when the cows were housed, and has been shown to be due to contamination from the byre floor. Tracy and Ramsey(80) described a malt flavour associated with the growth of a coccus. The taint was much more pronounced when a spore-former of the *B. subtilis* type was also present. The chief source of the rod form was found to be the utensils, whilst the coccus occurred in the udders of the cows in suspected herds. This coccus is believed to be a variety of *Staphylococcus aureus.* In a survey of the alkali-formers of milk, Henneberg(81) has studied a large number of strains isolated from milk and milk products. These organisms are protein-splitters and produce an alkaline reaction on China-blue agar. Seven main groups have been described and many well-recognised taints and off-flavours of milk are reported to be associated with their activities.

In reading through the recent literature relating to faults in milk and dairy products, a serious deficiency is evident, in that frequently no adequate bacteriological and ecological descriptions of the causative or associated organisms are given. This detracts from the value of much of the work, since the identity of the organisms cannot be established by subsequent workers. It is clear that the applied side of the subject has been developed at the expense of the more fundamental.

(*c*) Lactic acid bacteria.

The well-known difficulty of securing adequate growths of lactic acid bacteria appears to be largely a function of their nitrogen metabolism. Davis and Mattick(82) have demonstrated that the true lactic acid bacteria cannot grow in media in which the sole source of nitrogen is an amino-acid mixture or an ammonium salt. Further, whilst the streptococci grew best with those peptones containing the highest percentage of protein nitrogen, the lactobacilli grew best with those containing most amino nitrogen. Sadler *et al.*(83, 84) have studied the influence of 36 sources of nitrogen (casein digest, peptones, yeast preparations, etc.) on the sugar-fermenting capacity of five cultures of cocci isolated from cheese. The suitability of each was judged by the amount of titratable acidity produced by these organisms from glucose, mannose and lactose, using each source of nitrogen as the basis of the sugar broth. They found that the nitrogen distribution was not a sure index to the suitability of a source of nitrogen. For instance, in the broth prepared from two commercial peptones the nitrogen distribution was of the same order, but whilst one was very suitable for all the organisms studied, the other was far less satisfactory. Nevertheless, on the whole, the nitrogen distribution had a significant influence on the sugar-fermenting powers of the organisms, and the results showed that the amount of nitrogen was almost as important as the kind.

It is probable that the apparently discordant results of various workers may be attributed to caprice in the metabolism of the lactic acid bacteria. Weaver(85) has been unable to confirm the inability of *Lactobacillus bulgaricus* to ferment maltose, sucrose and laevulose, which has been taken as a distinguishing character between this organism and *L. acidophilus*. Weaver's methods showed greater precision than those of others, but he considered that the chief reason for his results was the use of a basic medium (casein digest broth, to which 0·15 per cent. agar and a combination indicator were added) which was well adapted for the growth of his cultures.

Various workers have confirmed the fact that lactic acid bacteria grow well on media containing plant extracts. Maddock(86) found that on bean agar containing 0·5 per cent. saccharose, *Str. paracitrovorus* gave an abundant slimy growth with a strong aromatic smell, quite distinct from that on ordinary nutrient media. Bachmann and Frost(87) used cabbage peptone agar for qualitative work with *L. acidophilus*. This was an easily prepared, clear medium, needing no adjustment, on which large colonies developed.

Genevois and Nicolaieff(88) demonstrated that lactic fermentation is specifically inhibited by α-halogenated aliphatic acids and that the ethyl esters and salts had a similar influence. Kantardjieff and Poppow(89) have studied the optical modification of the lactic acid produced by two strains of *L. bulgaricus*. Both strains gave *l*-lactic acid in milk, but *i*-lactic acid was produced in sugar broth and in beer wort by certain strains. This result is somewhat at variance with the observations of Orla-Jensen who uses the production of *l*-lactic acid as an important distinguishing feature in his classification.

The problem of the metabolism of this interesting group of organisms is clearly not yet solved.

III. PASTEURISATION OF MILK.

Pasteurisation of milk is a much discussed problem of the dairy industry. The widespread interest it has aroused is a comparatively recent development which has opened up a field for scientific research and application of mechanical skill. The last 40 years have been marked by a steady advance from the original flash pasteuriser which, owing to the thickness of the layer of milk treated and lack of adequate controls gave no certainty that all the milk was really heated as specified, and from the earlier types of the holder pasteuriser (again with no assurance that all the milk was heated at the specified temperature for the specified length of time) to modern plants with their equipment for the automatic control of temperature and rate of flow, and rigid separation of the held milk from the unheld. With the increase in the practice of pasteurisation of milk supplies many countries imposed legal standards. Whilst some countries, *e.g.* Denmark, recognised flash pasteurisation[1] as the official method, others, *e.g.* America and England[2], insisted on the holder process. Thus both methods have continued to be the subject of research.

The holder process has now reached a stage when there should be very little excuse for uncertainty that all the milk is really held at the stated temperature for the stated length of time. The installation of heat regenerative systems has resulted in a decreased steam consumption, and therefore an increased economy in working. Even so, this process is less economical of time and space than flash pasteurisation. Much of the recent research has therefore shown a tendency to revert to the principle

[1] Flash pasteurisation has been here defined as heating to at least 80° C. (176° F.) and immediately cooling.

[2] The holder process of pasteurisation infers heating at 145–150° F. (62·8–65·5° C.) for at least 30 min. and then cooling.

of flash pasteurisation, the object being to evolve a method which shall be rapid and economical, and yet at least as efficient in the destruction of bacteria as the holder process.

An essential point to be considered in judging a pasteurisation process is the efficiency of the destruction of bacteria, pathogenic organisms in particular. Commercial points cannot, however, be wholly excluded, and a very important factor is the customers' preference in many countries for a fresh flavour and a good cream-line. Flash pasteurisation at the usually accepted temperatures destroys the cream-line entirely, hence lower temperatures must be used in the ideal process. Recent investigations have led to the idea that the time of holding might be considerably shortened without loss of bactericidal efficiency if the temperature were suitably raised.

With the improvement in automatic control devices and the use of water as a heating medium, milk can be made to flow at a controlled rate in a very thin layer over or between heated surfaces so that its temperature can be rapidly adjusted and regulated. A type of apparatus that has attracted attention, especially in some European countries, is that devised by Stassano(90). The milk is heated to a temperature of 75° C. whilst flowing in a thin film, 1–1·25 mm. thick, in the annular space between two concentrically arranged pipes immersed in hot water in such a way that the film of milk is heated on both sides simultaneously. In some types the heat regenerative system consists of a series of metal tubes(91), but in later forms for treating larger quantities these have been replaced by a more compact battery of circular discs provided with spiral channels through which the milk or hot water flows(92), the tubes always being retained for the actual pasteurising process. In the words of the inventor, these tubes constitute the essential feature of the apparatus, for it is only when the milk flows in an extremely thin film between two heated surfaces that it can be assumed that the required temperature is reached instantaneously(93). This heating action has effect for 15–16 sec. whilst the milk is travelling a distance of 20 metres through the tubes.

This apparatus has been tested at Die Preussische Versuchs- und Forschungsanstalt für Milchwirtschaft, in Kiel(94), and at Statens Forsøgsmejeri, Hillerød(91), and both reports state that when the pasteurising temperature was adjusted to 75° C., *Bact. coli*, *Myco. tuberculosis* and *Br. abortus* (Bang) were all destroyed by the treatment. This method is now officially recognised by the Danish authorities under certain conditions, *e.g.* that the milk shall be heated to 75° C. for at least 15 sec. and immediately cooled to 12° C.(95). Chemical tests showed very little change in the composition of the milk, which is attributed to the fact that heating in closed tubes prevents the loss of CO_2. There was a very slight change in *p*H and an approximately 10 per cent. decrease in the rennetting capacity, whilst the vitamin A and B content was unaffected. On the other hand, the creaming ability was considerably reduced. According to the report from Kiel(94) it was almost completely destroyed, and the report from Hillerød(91) indicates that it was very seriously impaired by the treatment of the milk at 75° C. This is hardly in agreement with the claims made.

Other types of pasteuriser were also tested at Kiel, and were on the whole favourably reported on, but the greatest factor of safety, presumably in the destruction of pathogens, appeared to lie with the holder process. Amongst the flash pasteurisers were included various types of tubular and plate pasteuriser which latter originated and were perfected in England in 1923 for the pasteurisation of milk (British Patents 223,033, 255,109, etc.). These systems have a wide use as regenerative heaters and coolers forming units of plants set up for the holder process, but they are also adapted for use as flash pasteurisers, and appear to give very efficient service for this purpose.

Modern developments in England have led to the insertion of a holding section in some of these systems, by means of which milk can be held at the required temperature for short periods (12–20 sec.). The introduction of a holding period would appear to be a wise precaution.

A Plate Pasteuriser made by the Aluminium Plant and Vessel Company Limited (A.P.V. Phönix) was amongst those tested at Kiel(94). This was not built as a flash pasteuriser, having been intended merely as a heater and cooler in connection with the holding process, but gave good results bacteriologically when used at a temperature of 80° C. At this temperature the creaming ability of the milk was destroyed, but the report showed that the bacteriological effect was in the main good at 75° C., at which temperature a large proportion of the cream rose. Experiments made at the National Institute for Research in Dairying, Reading, with a machine specially adapted for pasteurising under these conditions and including special holding plates, has shown equally good results at still lower temperatures, with perfect creaming properties. These results are not yet available for publication. Tests were made on a similar apparatus at Statens Maskin- och Redskapsprovningsanstalter, Ålnarp(96), with similar results, but the holding section was not used, since it was found unnecessary for the achievement of one of the main objects of the investigation, viz. the determination whether the milk so treated reacted negatively to the Storch Test.

Lembke(97) has described bacteriological tests made with an Astra plate pasteuriser, which, if not identical with, closely resembles the above apparatus. He found that with the latest form of holder a working temperature of 70° C. would give a margin of safety for the destruction of resistant strains of *Bact. coli*. Drewes(98) records tests on the plate pasteuriser "Discus" in which the milk was heated for a maximum of 10 sec. at temperatures from 70 to 85° C. and immediately cooled. At 70° C. the author reports a reduction of plate count of 99·27 per cent., at 75° C. of 99·92 per cent., at 85° C. of 99·992 per cent. In the Kiel report on this apparatus(94) it is stated that the holding section had a significant influence only when the pasteurising temperature was 75–80° C. Above 80° C. the flash pasteurisation was so good that holding had no further effect, whilst at temperatures of about 70° C. the holding temperature was not sufficiently high to bring about any appreciable further reduction. In this connection the effect on numbers of the presence of thermoduric organisms may have to be considered.

Tests on various types of apparatus (*e.g.* Tödt, Discus, Montana, Grossphönix and Kleinphönix, the last two made to the design of the Aluminium Plant and Vessel Company Limited) are reported from Die bakteriologische Abteilung der Südd. Versuchs- und Forschungsanstalt für Milchwirtschaft in Weihenstephan(99). On the whole the reduction of the plate count and the destruction of coliform organisms in the milk appeared to be good when the samples were taken immediately after the heating process. The tests on samples taken after cooling showed a definite reinfection from the regenerating section in the case of the Phönix and Discus types. Demeter and Mossel(99) attributed this to contamination of this section by the first imperfectly pasteurised milk. This reinfection did not occur in the Montana pasteuriser which is provided with an outlet for the withdrawal of this first milk before it reaches the regenerating section. Their view is supported by the results obtained with the A.P.V. plate pasteuriser under test at Reading, to which reference has already been made, where the somewhat elementary condition of not putting imperfectly pasteurised milk through the cooling section has been observed. On the other hand, the Kiel report(94) records a reinfection with *Bact. coli* in the regenerative system of the Montana and Stassano machines.

Reinfection of milk may result from inefficient cleaning and sterilisation of the plant, but it would appear that in the reports already referred to little attention has

been paid to this side of the question, and control tests for sterility of plant have not been recorded. Many machines appear to be too intricate to allow of easy and certain cleaning and sterilisation and a criticism which might be levelled at Stassano's apparatus is that the dismantling and cleaning of the tubes involves a considerable amount of labour. It is claimed, however, that since CO_2 is retained in the milk by this particular method of heating there is little change in the calcium salts, and the deposit of milk stone is almost negligible and is easily removed by brushing, which should not be a difficult process for tubes of 10 cm. diameter. A thorough rinsing with cold water, soda water and hot water after the end of the run, with scalding or, "if desired," steaming, immediately before the beginning of the next run is considered sufficient to keep the plant in good working order, and it is stated that dismantling is necessary only at weekly or fortnightly intervals. These statements(91) appear to be based on experimental runs when the plant was not working at full pressure, and reports are not available of what would be the result of such treatment if the plant were working at full pressure under commercial conditions. To rely on methods such as these for the cleaning of a plant seems to the writers to be indefensible. The same remarks about the difficulty of cleaning would apply to all types of tubular heaters. A point in favour of the plate pasteurisers is the fact that they can be easily taken apart and cleaned after each run, and all parts are open for inspection.

In America, the mode of treatment termed "High Temperature-Short Time" pasteurisation has received considerable attention, and four types of apparatus have recently been examined. In one of these, the Electropure, the milk was heated by means of an electric current between two carbon electrodes in a small, specially designed chamber. The others were water-heated; the Isotherm and Precision were of the internal tubular type, the York Plate was of the multiple plate type made to English design. These have been reported on by Holmquist(100) but the tests were not exhaustive, neither were they complete in all cases. The Electropure and the York Plate pasteurisers were rather stringently tested for destruction of pathogenic organisms, especially *Myco. tuberculosis*, and it was found that a temperature of 153·9° F. for 12·4 sec. rendered tubercle-infected milk non-infectious for guinea-pigs. The stipulation which was laid down by the Health Department of the States of New York and Pennsylvania in accepting this method, that every particle of milk must be heated to a temperature of 160° F. for not less than 20 sec., should thus leave a good margin of safety.

Little information is yet available as to the extent of the chemical changes induced in milk by this method of heat treatment. Marquardt and Dahlberg(101) and Dahlberg(102) have studied the creaming ability of milk after various methods of heating. The results at 160° F. were rather irregular and the milk could only be heated for a very short time without affecting the cream layer. The relationship between the maximum temperature at which milk could be heated for varying periods of time without affecting the creaming ability could be expressed as a straight line on a semi-logarithmic graph. Dahlberg plotted in the same way the results that had been obtained by North and Park(103) for the thermal death periods for tubercle bacilli for temperatures from 132 to 212° F. Unfortunately, the figures above 155° F. were not suitable for comparison, since, except at 212° F., no samples were taken in less than 20 sec. These were all negative, so that the thermal death period was less than 20 sec. at these temperatures, but the actual figures were not determined. There was a very marked similarity between the two series of results when plotted on the same graph. From their figures North and Park concluded that "a standard of 142° F. for 30 min. is a proper standard for pasteurisation." From his comparative graphs Dahlberg deduced that on the basis of the destruction of

tubercle bacilli and the impairment of the creaming ability, heating at 160° F. for 16 sec. should give results comparable with heating at 142° F. for 30 min., a theory which agrees very well with practical findings. The length of time required to raise the temperature of the milk would also be a most important factor in determining the length of the holding period.

This "high temperature-short time" modification of the process of flash pasteurisation has aroused interest in many countries, but in only a few cases has it been officially recognised. In Denmark, where the flash process is the official method, the stassanising process is accepted(95). In two states of America the Departments of Health have approved of the Electropure, Isotherm, Precision and York Plate machines, and there is a movement in favour of recommendations that specifications for high temperature-short time pasteurisation at 160° F. for 15 sec. be included in the next edition of the United States Public Health Service Milk Ordinance and Code(104). Many problems are involved, and the effect of this form of heat treatment on the bacterial content and on the chemical composition of the milk has not yet been fully studied. The effort to use a very short holding time and a temperature which, whilst high enough to destroy the pathogenic bacteria, is low enough to ensure a good cream-line, leaves only very small margins of safety. The automatic control devices must be very efficient and reliable, and though they appear to be satisfactory for experimental quantities no figures are yet available for the use of this process on a large commercial scale.

The idea of pasteurisation of milk in bottles is constantly recurring, for such a method would eliminate the risk of recontamination. Compliance with the existing regulations for pasteurised milk is difficult, since it is not possible to state with certainty the temperature of milk within a closed bottle. In one system which is in use in Denmark(105) the bottles slowly pass through eight sections of a large circular vat. The cold milk is heated by means of hot water at 74° C. to a temperature of 63–64° C. in 20 min. It is maintained at this temperature for 30 min. and afterwards cooled by means of circulating cold water. Another method which is in use in some dairies in England consists in filling milk at a temperature of about 147° F. into hot bottles, then passing the capped or sealed bottles through a hot-air chamber maintained at 146–147° F. The milk is held at this temperature for 30 min., and then cooled to 40–42° F. in 31 min. by passing the bottles through a contra-flow of cold water entering at 36° F. Results of bacteriological tests on milk of average quality treated by this process show that the plate count was reduced to a very low figure and that *Bact. coli* was eliminated in each case (Enock(106)). The creaming ability was good and the milk had an exceptionally long keeping quality. The experimental trials of this method of treatment have given such satisfactory results as to merit close attention.

The efficiency of pasteurisation is to some extent governed by the quality of the raw milk. Hussong and Hammer(107), in controlled laboratory experiments on milk delivered at a dairy, found no close relationship between the initial counts and the pasteurisation efficiency. In some series, however, there was a tendency for high initial counts to be accompanied by high percentage reduction and *vice versa*. The wide variations in the results obtained for milk from different farms were presumably due to variations in the proportion of heat-resistant organisms in the flora. Thurston and Olson(108) similarly found that the efficiency of pasteurisation was generally lower for Grade A than for dirtier milk. This they attributed to a fairly constant number of resistant organisms in milk of any grade.

It is necessary that the efficiency of a pasteurising process shall be constantly controlled by means of bacteriological tests. The plate count is generally used, but McCrady and Langevin(109) have pointed out that this alone is not adequate, and

suggest that it should be supplemented with a test for organisms of the *coli-aerogenes* group, since these organisms are rarely found in efficiently pasteurised milk, and their presence is an indication of recontamination or survival in foam. This opinion is disputed by Beavens(110, 111), who found that although the "majority death-point" of a culture of *Bact. coli* may be 144° F. for 30 min., its "absolute death-point" may be raised to 148° F. for 30 min. His results also suggest that the lactose in milk may have a protective influence on these organisms. The views of McCrady and Langevin are supported by the work of Hoy and Newland(37) who found that milk taken direct from the holder almost invariably had a low plate count and high keeping quality, and was always free from coliform organisms. Contamination during the subsequent handling was shown in the increasing count and decreasing keeping quality and the striking reappearance of coliform organisms in the samples taken from subsequent parts of the plant. On the showing of the work of Beavens(110), even though some of the coliform organisms may resist the pasteurisation process, they will not be numerous and the imposition of a standard would serve as a check on excessive recontamination of the milk (Savage(112)).

Hansen(113) showed by feeding experiments with guinea-pigs that his strains of thermophiles were not pathogenic, and that pasteurised milk containing thermophiles is probably not harmful for human consumption. Their presence in large numbers, however, leads to deterioration of the quality of the milk and is an indication of faults in the management of the pasteurising plant or inefficient cleaning. The determination of the thermophile count is therefore an important factor in the control of pasteurised milk. Agar plates are generally used for this purpose, but Breed(114) recommends direct microscopical examination in addition. The presence of large rod-shaped organisms, in numbers exceeding those in the raw milk, indicates the presence of thermophiles. The work of House and Mattick(115) confirmed the opinion that thermophilic organisms were rare in clean raw milk. Although frequently very numerous in milk pasteurised by the holder process, they have, as yet, rarely been found in milk pasteurised by the high temperature-short time process. From the results of the examination of 100 samples Yale and Breed(116) showed that the conditions were unfavourable for their development.

Mudge and Thorwaldson(117) have advanced a new theory to explain the sudden rise and fall of the numbers of thermophiles during a long run in a pasteurising plant. They suggest that the thermophiles are present at first as dormant spores which germinate under the influence of heat (or other stimulus), grow rapidly for a short time and then resporulate. The high counts are those taken during the period of active vegetative growth. A measure of support is lent to this theory by the work of Christian(118), which showed that the germination of the spores of an organism, isolated from sterilised milk, was dependent upon a heating process.

Pasteurisation reduces the bacterial content but it does not always give a sufficient prolongation of the life of the milk to meet special requirements. Attempts have therefore been made to evolve processes which shall ensure a more nearly sterile product. To attain this object the treatment must be sufficiently severe to destroy all types of bacteria. The application of excessive heat causes serious chemical changes, and suggestions have been made to combine mild heat with other forms of treatment. In 1930, Williams and Gaines(119) reported that *Bact. coli* could be destroyed by holding broth suspensions above the source of sound waves. More recently Gaines and Chambers(120, 121) have investigated the action of such waves upon milk and have found that 80 per cent. of the organisms can be destroyed. Spores appear, however, to be able to withstand the treatment. The action of silver as a sterilising agent for milk has been examined by several workers. Demeter(122) claims that low-temperature flash heating followed by filtration through catadyne silver reduces the

number of bacteria, destroys the coliform organisms and enhances the keeping quality by 24–48 hours. This observation is interesting but does not seem to offer practical application. Krause(123) and Heine(124) using similar, but not identical, methods appear to have been able to confirm the improvement in keeping quality.

Pasteurisation of milk by means of electrical currents, although essentially a heat treatment, has also received attention. An apparatus is described by King(125) in which milk circulates between water-cooled carbon electrodes carrying a current of 220 volts. It is claimed that the temperature of the milk is raised to 72° C. with a minimum exposure to air and metals. Comparative tests have shown that electrical pasteurisation at 73° C. for 5 min. is as efficient in reducing the numbers and kinds of bacteria as the ordinary holder process at 62° C. (Stabler(126)), but it has the serious drawback of destroying the creaming ability and is possibly, like other electrical methods, expensive.

Improvements in the apparatus have brought the Jonas Nielsen(127) process into prominence again. Here the sterilising action is entirely due to high temperature, but the fact that the milk is heated in closed tubes under pressure is claimed to minimise the amount of chemical change.

The commercial process of sterilisation by heating at a temperature of 100° C. (212° F.) for 30 min. or more, frequently used in England, is rarely sufficient to destroy all spore forms. Hiscox and Christian(78) have shown that the survival of spores in this process may lead to considerable losses due to various forms of spoilage. To be really efficient, sterilisation of milk implies the destruction of all bacteria, including their spores, and at the present time no commercial methods, except perhaps those in which steam under pressure is used, can claim to be infallible in this respect.

IV. BUTTER.

For most purposes full-flavoured, slightly salted butter made from ripened cream is preferred, but a demand for unsalted sweet cream butter has arisen as a result of its use in the making of ice-cream mixes and reconstituted cream. The question of the development of flavour has been the subject of scientific research, and has directed the energies of the practical dairyman.

It has long been known that the use of a good starter is of primary importance in the production of a fine full-flavoured butter. A comprehensive review of the work that had already been done on starters was given in two historical surveys by Knudsen(24) and Kelly(128) which appeared almost simultaneously in 1931. A full account of the bacteria which constitute a good starter is given in each case, but some confusion as to nomenclature may arise from their perusal, since, for instance, Knudsen, following Orla-Jensen's system of classification of the lactic acid bacteria, refers to the aroma bacteria as *Betacoccus cremoris*, whilst Kelly uses the names *Streptococcus citrovorus* and *Streptococcus paracitrovorus*. In his studies of the lactic acid bacteria from starters Orla-Jensen(129) has distinguished between *Str. lactis* which produces only lactic acid by fermentation of lactose, and the closely allied form which produces small quantities of volatile acids and CO_2 in addition, and which he has called *Str. cremoris*. During the study of a large number of organisms from spontaneously soured milk and good commercial butter starters, Orla-Jensen and Hansen(130) were able to confirm the preponderance of *Str. cremoris* over *Str. lactis* in good butter starters, and also the characteristic differences in the fermentation of the di- and poly-saccharides by these two strains.

The term *Str. cremoris* as used by Orla-Jensen has not been universally adopted, and many workers still apply the term *Str. lactis* to the characteristic starter bacteria. Continuing work on the aroma bacteria, Farmer and Hammer(131) have referred to

Str. lactis, and have demonstrated that only certain strains will combine with *Str. citrovorus* and *Str. paracitrovorus* to yield satisfactory butter cultures. When a satisfactory combination is obtained this will give good cultures over a long period, provided the two types are kept in active condition. Eventually, the combinations of two types established the same proportion, irrespective of the comparative numbers in the original mixture, so that the same general flavour and aroma always developed. This point has been realised by practical dairymen, and, from experience in a large number of Danish dairies, it is reported(132) that too frequent renewal of butter starters is not favourable for the production of the highest grade butter. Stability is only obtained when the aroma bacteria peculiar to each dairy have established themselves in the culture. This is illustrated by the results of the butter tests at the Danish Dairy Shows during the last 6 years. The percentage of exhibits that gained silver medals was much higher for the dairies in which "individual" starters were used than for those using "laboratory" starters.

It is a well-known fact that the flavour of butter made from ripened cream is apt to deteriorate on storage, and Lucas *et al.*(133) were able to demonstrate experimentally that, although butter made from slightly ripened cream attained the highest score in the fresh state, it deteriorated during storage more rapidly than that made from sweet cream. The addition of starter at the time of churning did not apparently shorten the keeping quality of the butter. It would thus appear that the presence of lactic acid has an influence on the deterioration of butter. Following up the suggestion of Hammer(134) that, since the aroma bacteria produce only small quantities of lactic acid it might be possible, by the use of these organisms alone, to produce a full-flavoured butter which would not tend to deteriorate in the same way as that made from acid cream, Maddock(86) experimented in the use of pure cultures of *Str. paracitrovorus* as starters in butter-making. The butter was good, often of outstanding flavour, but the difficulty in maintaining the vigour of the cultures was so great as to preclude the recommendation of this practice as a commercial proposition.

It is now fully recognised that the aroma of ripened cream butter is due to the substances produced by the bacteria in the starter, the aroma bacteria in particular. The work of Van Niel, Kluyver and Derx(135) and of Schmalfuss and Bartmeyer(136) established that diacetyl is either the butter aroma itself or its chief component. Testoni and Ciusa(137) have suggested that its presence should be used as a test for fresh butter. On the other hand it has been suggested that diacetyl, or extracts from bacterial cultures containing it, might be used to improve the flavour of butter and margarine. Such a practice would, however, be illegal in many countries. Moreover, diacetyl is a very volatile substance which also tends to react with the fat, producing bleaching and oiliness in the butter. This reaction has been studied by King(138) and Tapernoux(139), who associate it with the deterioration, during storage, of butter which had a good aroma when freshly made.

The effect of light from different parts of the spectrum in causing rancidity of different foodstuffs has been studied by Coe(140), who has found that green light delays rancidity in fats. He recommends the use of green or black wrappers for butter as a means of lessening spoilage. In this connection it is interesting to note that as the result of experiments carried out in their laboratories, the makers of Hansen's Starters now use dark brown bottles for the carriage and storage of the starters(141). In a white bottle, exposed to sunlight, the starter acquired an undesirable metallic flavour, but no reaction could be detected after several hours' exposure in dark brown bottles.

Although a certain amount of deterioration of butter may be due to purely chemical changes, contaminating micro-organisms are nevertheless responsible for

many "off-flavours." Their influence is most marked in unsalted sweet cream butter, but they may also occur in ripe cream butter, especially if an unsatisfactory starter is used. Demeter and Maier(142) have studied the changes in the bacterial content of ripe cream butter during different periods of storage. They found that the extent to which the bacterial count increased or decreased during 16 weeks' storage bears a relation to the keeping quality; the greater the increase, the poorer the quality of the butter. They consider that microbiological control of butter should be of great practical value in advisory work and in the detection of the beginnings of deterioration. Hammer and Nelson(143) have developed a method for the microscopical determination of the number of micro-organisms in butter, in which a representative sample is melted by heating to 45° C. and centrifuged until the serum has separated from the fat. The serum is withdrawn, and 0·01 ml. spread over a definite area on a slide, stained and counted under the microscope. This method has the advantage of giving rapid results indicating also the nature of the flora.

Very frequently a knowledge of the type of organism is of greater value than the total number, in determining the quality of butter. Grimes(144) has pointed out that in determining the keeping quality of butter many factors, including the types of organisms, their symbiotic relationship and the influence of temperature, salt content, texture and acidity on their growth in butter must be taken into consideration. On the other hand Demeter and Maier(145), examining the mould and yeast count, number of acidifying, non-acidifying, and caseolytic organisms, and total count on casein agar of 500 samples of ripe cream butter, stored for 70 days at 38° F. before scoring and testing, found that the total count on casein agar alone gave indication of the keeping quality of the butter. Arup and Gilmour(146) tested at intervals, bacteriologically and chemically, samples of Irish Free State creamery butter which was stored at − 7° C. They could deduce no relationship between counts and flavour of the butter and concluded that flavour was due to type of organism, but that any objectionable flavour which developed during storage was due to chemical oxidation processes. The influences of chemical, physical and bacterial factors on the deterioration of butter are so closely linked that, in the present state of knowledge it is difficult to disentangle them. This may account for many apparently conflicting statements.

The yeast and mould content is considered to exert an influence on the quality of butter, and methods for determining the plate count of these organisms are important. White and Hood(147) have studied this problem and have shown that when the platings were made on dehydrated malt agar, a *p*H of 3·4–3·8 inhibited the growth of bacterial colonies but did not seriously affect the yeast or mould growth. A temperature of 25° C. with an incubation period of 5 days was considered the most satisfactory. Davis(148) has described a synthetic medium of almost constant composition which may be accurately adjusted to any given *p*H between 3 and 6, by the addition of a corresponding amount of citric acid found by calculation. The acidity of whey and beer wort may be adjusted in the same manner, but in this case a buffer mixture must be also added to eliminate inaccuracies resulting from variations in the composition of the whey and beer wort.

Moulds are frequently found on the surface of butter where they cause unsightly discolorations. It is comparatively rarely that they develop within the body of the butter. Walts(149) has studied the growth of *Penicillium roqueforti* on the surface and in cracks of butter, and Boekhout and van Beynum(150) have described a mould of the genus *Epicoccum* which caused red spots on butter. Moulds may be suppressed by the use of salt, but the latter is sensitive to 11 per cent. and some others require more, so that the salting of butter rarely inhibits mould growth, it only decreases it.

The parchment paper used for wrapping is often suspected of carrying mould

infection. Greger(151) has shown that the paper is sterile when made, and later contamination comes from the air. He considers that the influence of infection with mould spores should not be over-estimated; the chemical and mechanical properties of the paper should be specially considered. Further the type of mould spore is of greater importance than the number. Arup(152), in analysing a number of samples of parchment paper, nôted that those which gave a high figure for soluble extract supported mould growth. He recommends boiling in brine for the sterilisation of mould-infected paper, since the mere presence of salt in high concentration is not sufficient to kill some of the common moulds, notably *Cladosporium herbarum*.

On the other hand, the growth of bacteria is usually readily inhibited by salt, *e.g.* *Pseudomonas fluorescens* is restrained by 1 per cent. As Clayton(153) has pointed out, most defects in butter flavour due to bacteria may be restrained by 2·5 per cent.

A pure water supply is essential for the production of good quality butter. During an investigation of "surface taint" Schutt(154) found that all the creameries affected were using water infected with putrifying organisms, especially *Pseudomonas fluorescens*. Derby and Hammer(155), in investigating the same problem, suggested that the taint was not due to surface contamination of the butter but to contamination of the cream. It appeared to be due to a number of species of bacteria which brought about a definite type of protein decomposition. Salt and the use of starters should control it. The recent prominence of surface taint is probably due to the demand for low acid unsalted butter. Virtanen(156) has dealt more fully with the question of faults in butter due to the action of water bacteria. He found that the most important of these were *Ps. fluorescens* and "*Bact. punctatum*". These possess strong proteolytic as well as lipolytic enzymes which emerge from the living cell into the nutrient medium. This explains why the taint spreads throughout the butter and is not confined to the areas in which the bacteria develop. He found that the enzyme was unusually resistant to heat, tolerating a temperature of 90° C. for 10 min. Thus, if the organisms have had an opportunity of developing in the cream, pasteurisation will not prevent the development of the taint. A high acidity will inhibit its action however, so that the fault is rarely met with in ripe cream butter. Rumment(157) has pointed out that the butterfat is only slightly attacked in the cold, hence the butter undergoes very little change whilst in cold storage but acquires a tallowy flavour at higher temperatures.

Off-flavours may also be produced by bacteria of the *coli-aerogenes* group. Their development in butter has been studied by Hammer and Yale(158), who show that comparatively low temperatures and the presence of salt have a striking influence in restraining this group. The more rapid growth of bacteria of the *Bact. aerogenes* type may account for the high proportion often isolated from faulty butter, and indicates that they are of greater importance in the deterioration of butter than organisms of the *Bact. coli* type. This accords with the greater adaptability of *Bact. aerogenes* to saprophytic conditions.

It has been suggested by Knudsen and Jensen(159) that a determination of the catalase number of butter would provide a useful index to its keeping quality, but Virtanen(156) has questioned the usefulness of this test, although such bacteria as *Ps. fluorescens* and *Bact. aerogenes* produce catalase. The test would give no indication of the presence of their enzymes, and moreover, many organisms which produce catalase are harmless in the butter. Osols(160) has studied the problem in some detail and has shown that the catalase number is influenced by chemical substances, notably salt, and by the quantity of buttermilk remaining in the butter. He found that undesirable bacteria in milk frequently lowered the catalase number, whilst the lactic acid bacteria in some cases raised it, and he therefore concluded that the test was of little value for determining the quality of butter. If this is true it implies the

possession by lactic acid bacteria of catalase, a statement which is at variance with most previous cultural studies.

V. CHEESE.

Many of the problems connected with the cheese-making industry are intimately concerned with the activities of micro-organisms, and their solution often involves an accurate knowledge of the flora characteristic of the various kinds of cheese. It is therefore essential that the institution of control should be possible throughout the whole process of making and ripening of experimental cheeses. Golding(161), in a contribution to this problem, describes a cheese-ripening room with automatic controls for the regulation of temperature and humidity, which has been used successfully in the ripening of experimental Kingston cheese at a temperature of 60–65° F. and a humidity of 88 per cent.

The changes in the flora of cheese during ripening are studied by means of bacteriological examination at stated intervals. Burkey(162) advocates the use of sodium citrate instead of sand or other crystalline substances in preparing emulsions for bacteriological examination. A whole cheese may be used for analysis, or, as frequently happens, borings from one cheese may be taken for each examination. In the latter case the empty bore holes must be filled to restore, as far as possible, the former conditions. Wax plugs are generally used, but a method devised by Udy(163) for commercial purposes, in which finely mixed cheese is forced into the hole by means of a cheese "gun," might perhaps be adapted for this purpose if due precautions were observed.

In many cases the act of boring so changes the gas balance in the cheese that further observations are of doubtful value. Boekhout and Brouwer(164) have used Röntgen ray photographs in the study of the development of the holes in Gouda cheese. This method appears to offer great possibilities, and could, perhaps, be applied to other studies of the course of cheese ripening.

The rôle of the lactic acid bacteria in the process of cheese ripening has been a matter of debate. The first function of the lactic acid bacteria is to produce the degree of acidity required to ensure the correct texture of the curd and drainage of the whey. In the opinion of many, their activities end at this stage with the gradual disappearance of the living organisms, the degradation of the casein being carried on by the enzymes of the milk and rennet. Others have held the view put forward by Orla-Jensen that even after the lactic acid bacteria were dead their liberated enzymes played a part in the degradation of the casein, although it had not been proved by direct experiment. Virtanen(165), however, has now shown that milk cultures of *L. helveticus* to which chalk had been added, had a strong casein-splitting action even after the bacteria were dead. This proteolytic action could not be demonstrated with ordinary milk cultures, a fact which was found to be due to the rapid destruction of the enzymes at high acidities. Virtanen and Lundmark(166) found that if the *p*H of a culture did not fall below 4, a strongly proteolytic bacterial mass could be obtained, which, in the presence of toluene, would decompose casein as rapidly as a corresponding quantity of living cells. This work was carried out with cultures of *L. helveticus*, but it has been proved by Barthel that the lactic acid streptococci also contain casein-splitting enzymes. It was found that pure strains often induced less degradation of the casein than mixed starters(167). Kelly(168) compared the proteolytic action in milk of strains of *Str. lactis* and *Str. cremoris*. He found that the amount of amino nitrogen produced by *Str. cremoris* was approximately the same as that produced by a starter, whilst *Str. lactis* produced less than either. He regarded this as additional evidence that *Str. cremoris*, rather than *Str. lactis*, is the active proteolytic organism of starters. Later, however, during in-

vestigations of the ripening of Cheddar cheese he appears to have come to the conclusion that *Str. lactis* and *Str. cremoris* were equally suitable as starters (169) and states that in commercial cheese without starter and in experimental cheese with *Str. lactis* or *Str. cremoris* as starter, *Str. lactis* was found to increase until it constituted about 50 per cent. of the flora (170). During the ripening process of Cheddar cheese, the water-soluble nitrogen increased uniformly throughout the first 10–13 days, but even at the end of 85 days, 81·5 per cent. of the total nitrogen was still in the protein form (169). This is in agreement with the results of Virtanen (165) that the proteolytic enzymes of the lactic acid streptococci are either protease and polypeptidase or protease only, so that the casein degradation in cheese containing these organisms will not proceed so far as in those containing *L. helveticus*. Whilst comparing the course of the ripening of Cheddar cheese made from market milk and from clean milk of a low count, Allen (171) found a higher titratable acidity and a more extensive proteolysis in the former, probably because of the more rapid development of the lactic acid bacteria in a medium in which protein-splitting organisms have already grown.

A considerable amount of difficulty has arisen in the cheese-making industry as a result of "slow-working starters." Harrison and Hammer (172) have shown that from normal, rapidly coagulating, "quick working" pure cultures of *Str. lactis*, rapid and slow coagulating strains can be obtained. In general, the former induce considerable proteolysis, whilst in the latter it is very slight. The colonies obtained by plating a slow coagulating culture were all slow in their rate of coagulation. It thus appears that it is difficult to secure a rapid coagulating culture from a slow. Whitehead and Cox (173) have devised a test in which the vitality of a starter is determined by the rate of development of acidity in the whey which separates from the inoculated milk after rennet has been added.

The investigations of Tarnanen (174) and Virtanen (165) on the proteolytic enzymes of the lactic acid bacteria have thrown light on the ripening of Emmenthal cheese. The studies were carried out chiefly with *L. helveticus*, and demonstrated three proteolytic enzymes by means of which the casein was broken down to amino acids in three stages. These enzymes were sensitive to acidity and were destroyed if the *p*H fell below 4. Small differences in *p*H had a significant influence on the course of the enzyme action, and it is probable that many faults in cheese may be due to irregularities in the *p*H. Although the main course of the ripening of Emmenthal cheese is determined by the enzyme action of lactic acid bacteria such as *L. helveticus*, other bacteria are also involved. Studies on the "eye formation" by Frazier and Wing (175) have shown that "*B. acidi propionici*" does not usually increase in number until the third or fourth week when eye formation begins, but when eye formation is complete it usually predominates. The use of excess of "eye-former" culture resulted in an "overset" cheese, but when moderate quantities were used the number of eyes formed, the time of the beginning of eye formation and the completion of the set were independent of the number of bacteria added. Koestler and Hostettler (176) have found that the gas arising in the eyes during "after-fermentation" did not differ essentially from that in the eyes during the main fermentation, whence they concluded that the fault was due to a recurrence of propionic acid fermentation. The control lay in avoiding the recurrence of the factors causing the fermentation rather than in avoiding contamination.

The flavour of Gruyère cheese is to a large extent due to the penetration of ammonia formed on the surface by micro-organisms. This question has been studied by Demont (177), who has shown that several micro-organisms are concerned, but they all have the faculty of growing in a medium containing a high percentage of salt, such as is found on the exterior of the cheese.

Studies of the growth of *Penicillum roqueforti* have been continued in connection with the ripening of "blue-veined" cheese. Weisbrodt had shown that the substitution of NH_4Cl for $NaNO_3$ in synthetic media resulted in an increased growth of this mould. Golding(178) has, however, found that the addition of NH_4Cl to the curd at different stages of the manufacture of Wensleydale cheese in order to supplement the NH_3 normally produced during the ripening process, did not improve the rate or extent of the growth of the mould, but, on the contrary, exerted a retarding action. Assuming that the conditions most favourable for the formation and activity of the proteolytic and lipolytic enzymes of *P. roqueforti* are the same as those most favourable for the production of a Roquefort cheese of a good quality, Naylor *et al.*(179) have studied this problem. They found that the conditions which gave an increase in the weight of the mould growth fostered an increase in the esterase and protease activity, and that *p*H 5·30 was the optimum for proteolytic activity when a synthetic medium was used, but the results have not yet been directly applied to cheese-ripening. Hiscox(180) has studied the changes in the acidity and nitrogen distribution during the ripening of Stilton cheese. There was no indication of a direct relationship between the growth of the mould and changes in the titratable acidity or *p*H, but a significant decrease in the volatile acids appeared to be intimately connected with the development of the mould.

The identity of the white and blue strains of *Penicillium camemberti* has been confirmed by Leonidse(181), who suggests simplifying the nomenclature by giving the name *P. camemberti* to the blue strain, and *P. camemberti* var. *candidum* to the white. These two strains play the chief part in the production of the characteristic flavour and aroma of Camembert cheese, although the growth of *Oidium lactis* in combination with these moulds improves the reaction. Laxa(182) has studied the ripening of Ellishau cheese, which in most respects is very similar to that of Camembert. A new species of *Penicillium*, *P. Nalgiovensis*, closely resembling *P. album*, is important in the ripening, and is also responsible for the development of red pigment on the surface of the cheese. It produces a large proportion of amino acids and much ammonia and forms the red pigment by reaction with the alanine and tyrosine thus produced.

The whole problem of the classification and nomenclature of the moulds is still obscure. Laxa(182), working with strains of *Penicillium*, suggests that the growth on amino acids would provide a ready means of differentiation. Ritter(183), dealing with 174 strains of *Oidium* isolated from milk and milk products, divided them into four groups, incidentally studying their protein-splitting power. Grimes *et al.*(184), during the study of two species of *Phoma* isolated from dairy products, experienced the need for an improved method of classification and attempted to lay the foundation for a scheme based on the cultural, morphological and physical characteristics. In view of the great importance of many species of moulds in the dairying industry, a clearer knowledge of their relationships is necessary and would be of value in the interpretation of their reactions.

Further contributions to the knowledge of the flora characteristic of various types of cheese have been made by Diertelm's(185) and Grenz'(186) studies of the flora of normal Tilsiter cheese and Sacchetti's(187) studies of spore-bearing yeasts from Italian soft cheese. The flora of processed or pasteurised cheese has been examined by Csiszár(188). He found, in dealing with samples of experimental processed Emmenthal cheese and commercial processed Emmenthal and Trappist cheese, that the flora consisted mainly of lactic acid bacteria (both coccal and rod forms). Other organisms were also present, *e.g.* aerobic bacilli, butyric acid bacteria, other heat-resistant bacteria, and sometimes moulds and yeasts, the number and type depending on the age of the raw cheese and the time and temperature of heating during the

processing. He traced the "blowing" of processed cheese(189) to the presence of anaerobic spore-forming bacilli of the types *Cl. sporogenes*, *Cl. putrificum* and *Cl. butyricum*. The organisms which are the main cause of "blowing" in raw cheese are usually destroyed by the pasteurisation process.

The appearance of cheese is frequently spoiled by the growth of pigment-producing organisms on the surface. Bondioli(190) has isolated from the rind of a Grana cheese a coccus, an obligate aerobe, which gives colonies of an intensely red colour on lactose agar and other media. Stocker(191) has investigated the cause of black discoloration of the rind of soft and Tilsiter cheese. Usually this was due to the presence of *Monilia niger*, but in some cases it was traced to the growth of strongly aerobic bacteria, which produced a black or brown pigment at low temperatures. Demeter and Mossel(192) have studied the brown spot in Camembert cheese caused by *Penicillium bruneo violaceum* (Biourge), and concluded that the original source of infection was the rennet. They recommended that the rennet used should be free from yeasts and bacteria, and that the cellars should be disinfected and currents of air filtered. Moulds on the surface of Cheddar cheese may cause unsightly discolorations, and the work of Morgan(193) suggests that such surface growths may be responsible for bleaching and muddy discoloration in the interior. Rogers(194) has described a method of canning cheese direct from the press, whereby the growth of moulds on the surface is prevented by the exclusion of oxygen. The gases produced during the ripening of the cheese escape by means of a valve. Such a method could not, however, be applied to those types in which a surface ripening plays an important part.

Organisms of the *coli-aerogenes* group have a deleterious effect on cheese, large numbers tending to induce a gassy fermentation. Haglund *et al.*(195) have studied the problem in connection with Swedish cheese and have demonstrated that the addition of suitable quantities of starter will prevent the fermentation. Too large a quantity of starter will, however, spoil the texture of the cheese, but the quantity may be reduced if KNO_3 is also added, with consequently less influence on the texture. Salting, by suppressing the lactic acid bacteria, increases the tendency to gassy fermentation. Whitehead(196) has studied the influence of strains of organisms of the coliform group on the flavour of Cheddar cheese. When added to the milk immediately before the start of the cheese-making process, these bacteria did not produce gas holes if an active starter were used. It seemed probable that their metabolism was modified by association with the streptococci of the starter. Continuing these studies Cox and Whitehead(197) examined the influence of other bacteria on the production of acid by the lactic streptococci in milk. *B. subtilis* appeared to stimulate acid production, the action of *Bact. coli* was variable, staphylococci and *Bact. alkaligenes* had only a slight effect. The experiments are being repeated, adding the streptococcus to the milk at a later stage to simulate more closely the conditions in a cheese vat. Riddet *et al.*(198) have recorded a case of bitterness in cheese which appeared to be associated with the use of a slow-working starter, but the investigation is not complete. Mattick *et al.*(199) have shown that the most important factor in the growth and pigment formation of the organism causing rusty spot in Cheddar cheese is the presence of other bacteria in the milk, especially those which induce low oxidation-reduction potentials. The rusty spot could not be reproduced in cheese made from "clean" milk even when this was heavily inoculated with the causal organism, but it could readily be reproduced with commercial milk from various sources.

Hood and White(200) produced typical rancidity in experimental cheese by the introduction into the milk of butyric acid bacteria, milk cultures from rancid cheese, corn silage washings, or soil, etc., under various conditions. Their results showed that these undesirable bacteria may find their way into poor quality milk, but are rarely

found in milk of good quality. Many of the faults in cheese may be traced to the presence of contaminating bacteria which would not be found in milk of a high grade, and they could be eliminated by an improvement in the milk supply. In the meantime, pasteurisation has been resorted to as a method of removing these organisms. Whilst this process has been effective in improving the quality of cheese made with milk of only moderately good quality, it affects the course of ripening, as has been shown by Moir(201), and the flavour is not so good as it would have been had raw milk of a low bacterial content been used. Moreover, heat-resistant strains of bacteria, especially the spore-bearing bacilli, may survive the pasteurisation process and cause undesirable changes in the cheese made from such milk. For instance, Gratz(202) traced the swelling of Emmenthal cheese to the survival of *Cl. butyricum* in milk which had been pasteurised.

This review has been chiefly concerned with the activities of microscopic forms of organisms in milk and milk products, but recent researches have shown that ultramicroscopic forms may also have to be considered. For instance, Sherman *et al.*(203, 204, 205) claim to have shown primitive bacterial forms in soil, manure, hay infusions, faeces and milk. The work was extended to cover dairy products, in which the filterable forms were found in large numbers. They attached importance to the presence of these forms in aseptically drawn milk as suggesting a clue to the so-called germicidal action of raw milk, and in cheese as affording a possible explanation of the "death" of bacteria during the process of cheese ripening. Dorner's(206) results with Emmenthal and Gruyère cheese confirm those of Sherman.

Bacteriophage and germicidal action have also been studied, and the idea has been entertained of using coliphages to restrain the activity of coliform organisms in milk. Dorner(207) and Majer(208, 209) claim to have obtained positive results in this direction. Lipska(210), whilst unable to confirm this, has shown that freshly drawn and commercial raw milk may contain coliphages similar to those which occur in animal dejecta. The mode of action of bacteriophage and the so-called germicidal power of milk are not yet clearly understood, but it is possible that they and other similar actions may be factors in determining the nature and extent of the bacterial flora of milk. Recent work on bacterial life cycles, variation, filterable viruses, bacteriophage, lysozyme, etc., has shown that the life history of bacteria may be far more complicated than has been generally assumed.

The lack of knowledge indicates the urgent need for a closer study of the bacteria themselves, as individuals, quite apart from their industrial or medical significance. It is probable that the knowledge thus acquired may be of great service in the elucidation of industrial problems. The need for such study is further emphasised by the continual multiplication of species, with resultant difficulty of classification and confusion in nomenclature. For the sake of unity a system of nomenclature based on the classification of the American Society of Bacteriologists, 1920(211), has been adopted throughout this review, frequently necessitating the alteration of the names used in the original papers.

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THE TREND OF PRODUCTION AND TRADE IN MILK AND MILK PRODUCTS

In the world as a whole, milk is probably the most important single product in farming economy[1], and milk products play an important part in international trade. It is impossible to form even an approximate estimate of the aggregate milk supply, owing to lack of reliable data in many countries regarding both numbers of dairy cows and the average yield per cow. The following table gives an indication of the trend of the cattle population during recent years in countries which are of importance in the international trade in dairy products, whether as exporters or importers. Owing to the differences of classification of the cattle population as between "cows" and other cattle, and to the different dates on which estimates are made, the figures do not afford an exact comparison of the dairy herd between one country and another, but they do show the trend in each individual country.

Cattle population in certain countries.

Country	1929	1930	1931	1932
	000 head	000 head	000 head	000 head
United Kingdom: Total cattle	7,891	7,759	7,954	8,292
Cows	3,430	3,384	3,504	3,599
Irish Free State: Total cattle	4,137	4,038	4,029	4,025
Cows	1,307	1,311	1,300	1,297
Denmark: Total cattle	3,031	3,057	3,208	3,238
Cows	1,579	1,608	1,676	1,739
Norway: Total cattle	1,224	1,251	1,310	1,342
Cows	755	—	777	—
Sweden: Total cattle	—	3,060	3,109	3,120
Cows	—	2,033	2,037	2,043
Estonia: Total cattle	604	627	669	692
Cows	407	416	428	436
Latvia: Total cattle	978	1,026	1,117	—
Cows	—	—	—	—
Lithuania: Total cattle	1,160	1,170	1,120	—
Cows	692	696	693	—
Germany: Total cattle	18,033	18,470	19,124	19,123
Cows	10,356	10,442	10,592	10,815
Poland: Total cattle	9,057	9,400	9,786	9,457
Cows	5,968	6,191	6,551	6,571
France: Total cattle	15,631	15,467	15,434	—
Cows	8,196	8,288	8,274	—
Canada: Total cattle	8,825	8,937	7,991	8,511
Cows	3,685	3,683	3,513	3,725
U.S. America: Total cattle	56,389	59,730	60,915	62,407
Cows	21,849	22,910	23,558	24,379
Australia: Total cattle	11,202	11,721	—	—
Cows	2,465	2,631	—	—
New Zealand: Total cattle	3,446	3,770	4,081	4,072
Cows	1,371	1,441	1,602	1,702

In addition to the countries listed above there are a number of countries of importance in international trade in dairy products, but for which official annual estimates of the cattle population are not available. The number of cattle in the Netherlands, according to the census figures, totalled 2,366,000 in 1930. At the

[1] See *Dairy Produce*, EMB/C/1 (June, 1932), issued by the Empire Marketing Board.

Argentine census of 1930 the total number of cattle was returned at 32,211,000 and the number of cows at 13,745,000. The total number of cattle in Switzerland, according to a census taken in 1931, amounted to 1,609,000 and the number of cows to 971,000.

The increasing numbers shown by the foregoing table are part of a gradual upward trend in most of those countries. The increase is also due in part to a change over from other methods of farming, particularly the production of cereals, which felt the effects of falling prices earlier than did dairy products. This change in the farming economy has been especially noticeable in Canada and the Southern Hemisphere.

But milk production is the product of more than one factor, and the average yield obtained per cow is of particular importance. From the figures available it is evident that yields have increased steadily during post-war years, at least to the beginning of the recent period of low prices. Thus the average production of butterfat per cow in New Zealand rose from 182 lb. in 1924–5 to 218 lb. in 1929–30. In Denmark the average yield of milk per cow increased from 6826 lb. to 7011 lb. between 1924 and 1929, and the average yield of butterfat per cow from 251 lb. to 268 lb. The average lactation yield in England and Wales is estimated to have risen from 452 gallons in 1924–5 to 538 gallons in 1930–1.

This increased production has been reflected in the growing volume of dairy products entering world trade. The expansion has been most marked in two distinct areas, namely, in a number of Northern European countries and in the Southern Hemisphere. In the former group Denmark has steadily extended its milk production for half a century to establish firmly its position as the chief butter-exporting country. There has, however, been a more spectacular increase during the past decade in exports from the Secession States (Estonia, Latvia, Lithuania) and Poland, which modelled their dairying industry on Danish lines and assumed a significant place in world trade in butter. The main factors contributing to the increase in the Southern Hemisphere, particularly Australia and New Zealand, have been the opening up of new areas suitable for dairying, improved productive capacity per farm and per cow, a more recent change over from other types of farming, and favourable weather conditions in the past few years.

The following table illustrates the development of exports of butter, the principal milk product entering world trade, from the chief exporting countries during the four years 1929 to 1932.

Exports of butter from principal exporting countries.

	1929 000 lb.	1930 000 lb.	1931 000 lb.	1932 000 lb.
Denmark	350,620	372,558	378,429	347,886
Baltic countries*	102,123	114,573	119,818	93,216
Russia	56,097	23,149	68,024	68,198
Scandinavian countries†	92,778	96,819	83,041	64,316
Netherlands	104,325	92,394	72,660	44,926
New Zealand	183,639	209,771	222,718	244,588
Australia	102,919	126,411	191,016	229,105
Argentina	37,547	48,866	51,167	55,973

* Estonia, Latvia, Lithuania and Poland. † Norway, Sweden and Finland.

It will be noted from the foregoing table that a peak in exports appears to have been reached for several countries about 1931. The fall in prices did not affect dairy products until comparatively late. In 1931, however, a rapid decline did occur, butter prices in London, for example, being on the average 13–15 per cent. lower than in 1930. Lower prices were bound to be reflected sooner or later in diminished

production, and although no statistics of milk production in most countries are available for 1932, there is reason to believe that production was tending to decline, at any rate in European countries. There are no indications of any reduction, or even of any check to the rate of increase, of the dairy herds, but the depression began in 1932 to have some effect upon the average milk yield per cow. Less concentrates were fed, economies were made in manuring of pastures and in other operations in which heavy costs were involved. In Australia and New Zealand, however, production continued to expand.

Lower prices in external markets also led to more milk and milk products being consumed in the countries of origin, and exports, as a consequence, showed a greater proportionate decline than production. But factors other than the normal forces of supply and demand had also commenced to play a more important part—particularly political action. Growing imports were viewed with alarm by countries which, like France, had a large home industry and had figured as a net exporter of butter and cheese until comparatively recently, or, like Germany, desired to become more self-supporting and assist in preserving an active trade balance. Legislative measures were adopted and the quota method of control came into operation. The departure of the United Kingdom from the gold standard, accompanied by the depreciation in currencies of countries normally large exporters of dairy produce, such as Denmark, Sweden and the Southern Dominions, accentuated the difficulties in importing countries and hastened the adoption of restrictive measures. By 1932 the control of imported dairy produce by quota in one form or another was in operation in all the principal European importing countries, although in France it was temporarily replaced by considerably increased tariffs.

In the United Kingdom the imposition of tariffs in 1932, with free entry for Dominion produce, was less drastic than in most other countries. As a result, while world butter exports in 1932 were approximately 5 per cent. less than in 1931, the quantity taken by the United Kingdom increased by 6 per cent., and comprised about 77 per cent. of the total quantity entering world trade, against about 70 per cent. in 1931. The price of butter in the United Kingdom dropped by a further 8–10 per cent. between 1931 and 1932, and consumption of butter in the latter year is estimated at 21·8 lb. per head, against 20·9 lb. in 1931 and an average of 15·6 lb. in 1924–7.

These developments are illustrated in the following table, which gives imports of butter into the four chief importing countries in the period 1929–32.

Imports of butter into principal importing countries.

	1929	1930	1931	1932
	000 lb.	000 lb.	000 lb.	000 lb.
United Kingdom	716,492	764,782	903,967	946,298
Germany	296,230	293,560	220,950	153,264
France	9,753	12,924	40,836	26,140
Belgium	9,559	22,412	41,562	46,778

Similar considerations apply to cheese as to butter. There is bound to be a fairly close relationship between butter and cheese, due to the possibility of a change over from one product to the other.

The volume of cheese entering world trade, which had varied only slightly in earlier recent years, declined appreciably in 1931 and 1932. As in the case of butter, the reduction principally affected European countries. Decreased purchasing power in the importing countries of Europe and the United States combined with legislative control of imports to reduce exports considerably, particularly of the fancy types produced, for example, in Switzerland and Italy.

The following table gives exports of cheese from principal exporting countries and imports to the chief markets in the four years 1929–32.

International trade in cheese.

	1929	1930	1931	1932
Exports:	000 lb.	000 lb.	000 lb.	000 lb.
Netherlands	211,237	206,739	190,460	170,061
New Zealand	197,552	201,863	183,271	163,980
Canada	92,945	66,955	84,790	86,940
Italy	71,803	80,974	88,948	66,399
Switzerland	69,737	65,925	54,307	43,700
Imports:				
United Kingdom	335,331	348,592	323,091	336,733
Germany	146,570	137,459	120,404	108,688
U.S. America	76,382	68,313	61,992	55,032
Belgium	46,456	51,106	49,600	45,660
France	52,173	65,524	82,810	52,267

In so far as the United Kingdom is concerned, the cheese trade is mainly in the hands of the home producer, New Zealand and Canada. Prices dropped about 24–27 per cent. between 1930 and 1932, a decline corresponding to the drop in butter values, although imports showed little change in volume. Between 1931 and 1932, however, the decline was practically negligible and available supplies were light. This advantage over butter caused dual purpose factories in New Zealand to go over entirely to cheese during the height of the 1932–3 season. Simultaneously, there was estimated to be a large winter make of cheese in Great Britain. Early this year markets weakened under the increased supplies, and the bulk of the relative advantage over butter was lost.

From the foregoing considerations the following conclusions emerge:

(1) There has been a marked increase in milk production during the present century and an even greater relative increase in the quantity of butter entering world trade. With gradually increasing standards of living and normal conditions the growth of production is likely to continue.

(2) More recently, low prices in world markets have reduced the external trade in butter and cheese and have tended to check milk production.

(3) In the latter period the Southern Hemisphere has gained ground at the expense of Europe.

(4) The smaller international trade in butter has not yet been clearly reflected in imports into the United Kingdom, due partly to larger Australian and New Zealand supplies and partly to the restrictions on imports being less severe than in other importing countries.

(5) As a consequence, consumption of butter in the United Kingdom rose from an average of 15·6 lb. per head in the period 1924–7 to 21·8 lb. in 1932.

(6) Cheese, the demand for which is more inelastic, has experienced a corresponding fall in price, although imports have remained fairly constant.

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